Northern Spotted Owl (*Strix occidentalis caurina*) Genome: Divergence with the Barred Owl (*Strix varia*) and Characterization of Light-Associated Genes

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Accepted: August 22, 2017

Data deposition: This Whole Genome Shotgun sequencing project has been deposited at DDBJ/ENA/GenBank under the accession NIFN00000000. The version described in this article is version NIFN01000000. Specimen Sequoia blood sample deposited as CAS:ORN:98821, California Academy of Sciences, San Francisco, California, United States. *Strix occidentalis caurina* raw genomic DNA sequences obtained from CAS:ORN:98821 are available from NCBI Sequence Read Archive (SRA) (SRA run accessions SRR4011595, SRR4011596, SRR4011597, SRR4011614, SRR4011615, SRR4011616, SRR4011617, SRR4011618, SRR4011619, and SRR4011620). *Strix varia* raw genomic DNA sequences obtained from CNHM<USA-OH>:ORNITH:B41533 are available from NCBI SRA (SRA run accessions SRR5428115, SRR5428116, and SRR5428117). Program ScaffSplitN50s deposited at Zenodo http://doi.org/10.5281/zenodo.163683 and available from https://github.com/calacademy-research/ScaffSplitN50s. Program dupchk deposited at Zenodo http://doi.org/10.5281/zenodo.163722 and available from https://github.com/calacademy-research/dupchk. Program GItaxidIsVert deposited at Zenodo http://doi.org/10.5281/zenodo.163737 and available from https://github.com/calacademy-research/GItaxidIsVert. Program scafSeqContigInfo deposited at Zenodo http://doi.org/10.5281/zenodo.163748 and available from https://github.com/calacademy-research/scafSeqContigInfo. Program scafN50 deposited at Zenodo http://doi.org/10.5281/zenodo.163739 and available from https://github.com/calacademy-research/scafN50. Additional scripts deposited as NSO-genome-scripts at Zenodo http://doi.org/10.5281/zenodo.805012 and available from https://github.com/calacademy-research/NSO-genome-scripts. Gene and repeat annotation files, the raw variant call file, alignments of light-associated gene orthologs as well as assemblies of transcriptome sequences deposited at Zenodo http://doi.org/10.5281/zenodo.822859.

Abstract

We report here the assembly of a northern spotted owl (*Strix occidentalis caurina*) genome. We generated Illumina paired-end sequence data at 90× coverage using nine libraries with insert lengths ranging from ~250 to 9,600 nt and read lengths from 100 to 375 nt. The genome assembly is comprised of 8,108 scaffolds totaling 1.26×10^9 nt in length with an N50 length of 3.98×10^6 nt. We calculated the genome-wide fixation index (F_{ST}) of *S. o. caurina* with the closely related barred owl (*Strix varia*) as 0.819. We examined 19 genes that encode proteins with light-dependent functions in our genome assembly as well as in that of the barn owl (*Tyto alba*). We present genomic evidence for loss of three of these in *S. o. caurina* and four in *T. alba*. We suggest that most light-

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associated gene functions have been maintained in owls and their loss has not proceeded to the same extent as in other dim-light-adapted vertebrates.

Key words: nuclear genome, bird, Strigidae, Strigiformes, Aves.

Introduction

The spotted owl (*Strix occidentalis*) is a large, charismatic inhabitant of dense forests whose range extends along the Pacific coast of North America from southwestern British Columbia to southern California and eastward into the southwest desert states and Mexico. The northern spotted owl subspecies, *S. o. caurina*, inhabits the Pacific Northwest portion of the *S. occidentalis* range from British Columbia south along the west coast to the Golden Gate strait, California. The US Fish and Wildlife Service listed *S. o. caurina* as "threatened" under the Endangered Species Act (ESA) in 1990 (Thomas et al. 1990) and the owl has been the subject of much ecological research and economic tension. Since its listing under the ESA, populations have continued to decline (Forsman et al. 1996, 2011; Dugger et al. 2015; Davis et al. 2016) despite the increased level of protection. Although it is not considered a "model species" by most researchers, there is a considerable amount of demographic and ecological data available for this species (Courtney et al. 2004), especially in comparison with other owls, which tend to be less studied than diurnal birds.

Spotted owl conservation efforts often focus on genetic challenges, including those relating to small population sizes and inbreeding, relationships to other population segments, and potential interbreeding with congeners (Barrowclough et al. 1999, 2005, 2011; Haig et al. 2001, 2004). A complete genome assembly could provide many useful tools for conservation geneticists, including independent estimates of effective population size (N_e), tools for identifying and developing genetic markers such as single nucleotide polymorphisms and microsatellites, and data that can provide direct and relatively accurate measures of interbreeding.

The congeneric barred owl (*Strix varia*), formerly native to North America east of the Rocky Mountains (Mazur and James 2000), has invaded western North America in the last 50–75 years and, from British Columbia to southern California, has become broadly sympatric with the spotted owl in the last 50 years (Taylor and Forsman 1976; Livezey 2009a, 2009b) and likely poses a threat to the survival of the northern spotted owl (Forsman et al. 2011; Wiens et al. 2014; Dugger et al. 2015; Diller et al. 2016). In addition to competing for western forest habitat, barred and spotted owls interact at the genetic level as they can hybridize and successfully backcross (Haig et al. 2004; Kelly and Forsman 2004; Funk et al. 2007). Much of our motivation to assemble the northern spotted owl genome was to provide a resource to aid those studying the genetics of this owl and related taxa. Thus, we included analyses of the genome of a barred owl from eastern North America as a baseline comparison to the spotted owl. We compared genome-derived estimates of N_e from both species and calculated F_{ST} between them.

Access to high-coverage, relatively complete genomes also allows researchers to address questions that, without this resource, are inaccessible or difficult to answer. For example, previous work has suggested that owls have evolved an atypical avian visual system with high numbers of dim-light-adaptive rod photoreceptors (Fite 1973; Bowmaker and Martin 1978) and a diminished capacity for color vision (Bowmaker and Martin 1978; Wu et al. 2016). Whole genome sequencing can establish what mutation(s) or genomic rearrangements resulted in their reduced color vision and, with multiple genomes, one may test whether such mutations are lineage-specific or inherited from a common ancestor. The genome assembly of the barn owl (*Tyto alba*; Aves: Tytonidae) was available and allowed us to test owl-lineage-based hypotheses, but it was one of the lower-coverage, less complete of the available avian genome assemblies (Zhang, Li B, Li C, et al. 2014). A complete spotted owl genome, in addition to providing whole genome data for a representative of Strigidae, the other of the two families of owls, could also enable a definitive search for genes involved in nocturnal visual adaptations and a better understanding of the processes of mutation that lead to such adaptations.

Materials and Methods

Genome Sample

We collected blood from a captive adult northern spotted owl (*S. o. caurina*) at WildCare rehabilitation facility in San Rafael, California. The captive owl, named Sequoia and referred to as such hereafter, patient card No. 849, was admitted to WildCare on 5 June 2005 as an abandoned nestling found in Larkspur, Marin County, California (CAS:ORN:98821; table 1). We chose to sequence the genome of this individual as *S. occidentalis* is known to hybridize with *S. varia* (Haig et al. 2004; Kelly and Forsman 2004; Funk et al. 2007) and we wanted to ensure that we were sequencing the genome of a nonhybrid, nonintrogressed individual. The first Marin County *S. varia* detections occurred in 2003 and researchers estimated a population size of only three individuals by 2005 (Jennings et al. 2011). First generation hybrid individuals are phenotypically diagnosable with intermediate plumage characteristics (Hamer et al. 1994). Thus, if Sequoia had any *S. varia* genetic material, it would likely have been a first

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Table 1
Specimen Data

Specimen	County	State	Country	Date	Specimen Institution
CAS:ORN:98821	Marin County	CA	United States	26 Jun 2005	California Academy of Sciences
CNHM<USA-OH>:ORNITH	Hamilton County	OH	United States	29 Nov 2010	Cincinnati Museum Center

Note.—Information regarding the *S. o. caurina* and *S. varia* individuals from which we obtained genomic sequences for this study including the county, state, country, and date of collection for each specimen as well as the specimen code and institution where each specimen is archived.

generation hybrid and easily diagnosable as such. No plumage or behavioral features, such as vocalizations, suggested that it was a hybrid individual.

DNA Isolation

For genomic DNA libraries that required very high molecular weight DNA, we isolated DNA by using the precipitation method provided by the Gentra Puregene Kit (Qiagen, Netherlands) and following the manufacturer's protocol. We also isolated DNA using a column-based method, the DNeasy Blood & Tissue Kit (Qiagen, Netherlands), and used this DNA for those libraries where very high molecular weight was not essential. We assessed the quality and concentration of all isolated DNA using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, USA), 2100 BioAnalyzer (Agilent Technologies, USA), Qubit 2.0 Flurometer (Invitrogen, USA), and by running the DNA on a 1% agarose gel. We determined that the resulting DNA from both methods had high molecular weight with most of the DNA comprising fragments >50,000 nucleotides (nt) in length.

Illumina Data

We obtained paired-end Illumina data from nine whole-genome libraries constructed using a variety of methods with a range of average insert lengths from 247 to 9,615 nt. In our library construction we utilized a range of DNA shearing methods including enzyme-based, ultrasonication, and hydrodynamic forces using a Hydroshear DNA Shearing Device (GeneMachines, USA). We amplified all but one of the libraries using polymerase chain reaction (PCR) and sequenced them with read lengths from 100 to 375 nt (see supplementary table S1, Supplementary Material online; supplementary section 1.1–1.8, Supplementary Material online).

Trimming, Merging, Error-Correction

We trimmed the Nextera mate-pair data using the software NxTrim version 0.2.3-alpha (O'Connell 2014; O'Connell et al. 2015) (supplementary section 1.9.1, Supplementary Material online) in order to classify reads of mate pair libraries as true mate pair reads, paired-end reads, or singleton reads. We then removed adapters and low quality bases separately for the resulting mate-pair sequences, paired-end sequences, and singleton sequences using Trimmomatic version 0.32 (Bolger et al. 2014) (supplementary section 1.9.2, Supplementary Material online). We also used Trimmomatic to remove adapters from all non-mate-pair libraries (supplementary section 1.10.1, Supplementary Material online). In order to test how various trimming methods affected the assembly outcome, we trimmed to different thresholds for some of our preliminary assemblies by changing the Trimmomatic version 0.32 (Bolger et al. 2014) average quality score parameters. We did not apply the error-correction process to reads trimmed to a stringent quality threshold. For some preliminary assemblies, we performed adapter and quality trimming, but did not merge overlapping paired-end reads (supplementary section 1.13, Supplementary Material online). However, since substantial portions of the paired-end reads from all of the libraries, except the Nextera700 bp library, were overlapping, for the sequences that we used to generate our final assembly we joined overlapping paired reads using BBMap version 34.00 (Bushnell 2014) (supplementary section 1.10.2, Supplementary Material online). We then performed quality trimming on the non-mate-pair library data using Trimmomatic version 0.32 (Bolger et al. 2014) (supplementary section 1.10.3, Supplementary Material online). Since we trimmed using the relatively lenient threshold of trimming the read when the average quality over 4 bp dropped below quality score (Phred) 17, we next used the k-mer-based error corrector in the SOAPdenovo2 toolkit, SOAPec version 2.01 (Luo et al. 2012), to correct sequence errors (supplementary section 1.11, Supplementary Material online). For any read that became unpaired due to the loss of the paired read we separately subjected it to the same adapter, quality trimming, and error-correcting steps as the reads that remained paired (supplementary section 1.12, Supplementary Material online).

Genome Size

In order to estimate the *S. occidentalis* nuclear genome size from our Illumina data, we ran Preqc (Simpson 2014) with the paired-end sequences from the Nextera700 bp data set (supplementary section 1.14, Supplementary Material online).

Assembly

We assembled the *S. occidentalis* genome using SOAPdenovo2 version 2.04 (Luo et al. 2012). In order to determine the optimal assembly parameter options, we performed numerous trial runs experimenting with different k-

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mer values and parameters. We utilized the insert size estimated in the output of trial assemblies to refine our estimation of the insert sizes for our libraries and used these refined values as input into subsequent assembly configuration files (supplementary table S1, Supplementary Material online). After optimizing the SOAPdenovo2 assembly options, we generated fourteen further preliminary assemblies to test how using differently filtered versions and subsets of our Illumina sequence data affected the assembly outcome. We examined how the assembly was affected by trimming our data to multiple quality thresholds, using or not applying error correction, not merging or merging our overlapping paired-end data, assembling with different k-mers, using or not using singleton data, and dropping certain libraries (supplementary table S2, Supplementary Material online). We used dupchk (Henderson and Hanna 2016a) to check for sequence duplication in each sequenced library and found an elevated level of duplication in the Hydroshear library data, so we excluded all sequences from this library from several assemblies (supplementary section 1.15, Supplementary Material online).

Preliminary Assembly Assessment

In order to compare our preliminary assemblies, we removed contiguous sequences (contigs) or scaffolds less than or equal to 300 nt with the intent of removing any unassembled reads from the assembly. We calculated the contig and scaffold N50 as well as the number of scaffolds in various length classes using scafN50 (Henderson and Hanna 2016d). We calculated the total length of the assembly, the percentage of "N" characters in the assembly that represent sequence gaps between contiguous sequences joined by paired-end or mate-pair data (% N's), and the total number of scaffolds using scafSeqContigInfo (Henderson and Hanna 2016e). We were conservative in the calculation of these metrics and separated scaffolds into contigs at each N in the sequence. We then used CEGMA version 2.5 (Parra et al. 2007) to annotate a set of highly conserved eukaryotic genes (CEGs) in our assembly and thereby obtain an assessment of the quality and completeness of each assembly (supplementary section 1.16, Supplementary Material online).

We found it useful to assess the genome assembly's continuity and completeness at each stage of the assembly process. We searched for CEGs using CEGMA to evaluate our earlier assemblies. However, at this time, one of the CEGMA tool authors recommends that researchers use BUSCO in place of CEGMA (Bradnam 2015). Since we used CEGMA to evaluate our earliest assemblies, we continued to use CEGMA for continuity. We ran BUSCO on our final assembly and the results suggested similar completeness as those of CEGMA.

Determination of Final Assembly

We examined multiple statistics in choosing our final assembly. We valued high contig and scaffold N50 values, low % N's in the sequence, a low total number of scaffolds, larger numbers of scaffolds longer than 1 mega nucleotide (Mnt), and completeness as reflected in the number of conserved genes found by the CEGMA pipeline. We decided that the assembly that had the best statistics across these categories was assembly 4 (table 2) and proceeded forward with this assembly.

We filled gaps in the assembly using the gap closing tool in the SOAPdenovo2 toolkit, GapCloser version 1.12-r6 (Luo et al. 2012). The gap-closed assembly contained many sequences under 1,000 nt in length, a substantial portion of which appeared to be unassembled reads. We used ScaffSplitN50s (Henderson and Hanna 2016c) to compare statistics describing the continuity of the assembly after removing contigs/scaffolds of lengths 300, 500, and 1,000 nt as well as when using N blocks of lengths 1, 5, 10, 15, 20, and 25 to separate contigs within scaffolds. We decided to remove all contigs and scaffolds <1,000 nt for downstream analyses and will refer to the resulting assembly as "StrOccCau_0.2" hereafter (supplementary section 1.18, Supplementary Material online).

Final Assembly Statistics

We calculated basic statistics on StrOccCau_0.2 using the "assemblathon_stats.pl" script, which was used for comparison of the Assemblathon 2 genome assemblies (Bradnam et al. 2013). We used both CEGMA version 2.5 (Parra et al. 2007) and BUSCO version 1.1b1 (Simão et al. 2015a, 2015b) to annotate sets of CEGs and thereby assess the assembly's completeness (supplementary section 1.19, Supplementary Material online). We also calculated basic statistics and ran CEGMA as described earlier for other available avian genomes, including the barn owl (*T. alba*) (Zhang, Li, Gilbert, et al. 2014a; Zhang, Li C, et al. 2014), downy woodpecker (*Picoides pubescens*) (Zhang, Li, Gilbert, et al. 2014b; Zhang, Li C, et al. 2014), zebra finch (*Taeniopygia guttata*) (GenBank assembly accession GCA_000151805.2; Warren et al. 2010), bald eagle (*Haliaeetus leucocephalus*) (Warren et al. 2014; Zhang, Li C, et al. 2014), golden eagle (*Aquila chrysaetos*) (GenBank assembly accession GCA_000766835.1; Wesley Warren et al. 2014), chimney swift (*Chaetura pelagica*) (Zhang, Li, Gilbert, et al. 2014c; Zhang, Li C, et al. 2014), and chicken (*Gallus gallus*) (GenBank assembly accession GCA_000002315.3; Warren et al. 2017).

Contamination Assessment

To assess whether any assembled contigs were derived from contaminant nonvertebrate organisms, we performed a local alignment of all sequences in StrOccCau_0.2 to a copy of the

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Table 2

Metrics of Preliminary Assemblies

Assembly	contig N50 (nt)	scaffold N50 (nt)	Total Length of Assembly (Gnt)	Ns (%)	Total Number of Scaffolds	Number Of Scaffolds > 1 Mnt In Length	Partial CEGs Found by CEGMA	Complete CEGs Found by CEGMA
1	9,499	3,869,235	1.275	4.77	51,843	292	231	205
2	12,096	3,522,724	1.274	4.40	48,264	295	233	205
3	10,425	4,007,375	1.272	4.88	47,075	0	226	200
4*	**13,983**	**3,919,460**	**1.275**	**4.26**	**47,900**	**303**	**235**	**221**
5	10,315	4,164,870	1.272	4.45	46,146	287	232	206
6	9,142	3,780,867	1.275	4.86	51,615	296	230	202
7	9,802	3,478,271	1.274	4.42	54,240	327	233	209
8	12,650	3,665,028	1.271	4.18	43,092	313	231	204
9	12,006	3,587,241	1.271	4.66	44,939	307	226	201
10	12,487	3,586,666	1.271	4.26	44,345	314	232	204
11	14,651	3,917,141	1.276	4.26	50,636	293	234	217
12	14,627	3,728,521	1.276	4.28	50,349	305	234	219
13	14,672	3,917,121	1.276	4.26	50,129	293	234	217
14	13,967	3,431,044	1.300	4.50	127,384	318	238	218

NOTE.—Various continuity and completeness summary statistics for our preliminary assemblies. We removed contigs/scaffolds <300 nt in order to remove unassembled reads from the assemblies before calculating these statistics. We defined contigs with the very restrictive parameter that each N split a scaffold into a separate contig. "Partial CEGs found by CEGMA" refers to the number of gene sequences found by CEGMA in the assembly in at least partial completeness out of 248 total CEGs. An asterisk and bolded font mark the preliminary assembly that we chose to use as the basis for the final assembly.

NCBI nucleotide database "nt" (Clark et al. 2016; NCBI Resource Coordinators 2016) using NCBI's BLAST+ version 2.3.0 tool BLASTN (Altschul et al. 1997; Camacho et al. 2009). We searched for nonvertebrate hits in the top aligned sequences using a local copy of the NCBI taxonomy database (ftp://ftp.ncbi.nlm.nih.gov/pub/taxonomy; Clark et al. 2016; NCBI Resource Coordinators 2016) and GItaxidIsVert (Henderson and Hanna 2016b). We re-examined those sequences where any of the five output alignments was an alignment to a nonvertebrate using the web version of NCBI's BLAST+ version 2.4.0 tool BLASTN (Altschul et al. 1997; Camacho et al. 2009). We used bioawk version 1.0 (Li 2013b) to remove contaminant scaffolds from the assembly and will refer to the resulting assembly version hereafter as "StrOccCau_1.0." We calculated basic statistics on StrOccCau_1.0 using the "assemblathon_stats.pl" script (Bradnam et al. 2013) (supplementary section 1.20, Supplementary Material online). We confirmed that no CEGs were present in the contaminant scaffolds.

Mitochondrial Genome Identification

We searched for any contigs or scaffolds that were assemblies of the mitochondrial genome, rather than the nuclear genome by aligning a mitochondrial genome assembly of the brown wood owl (*Strix leptogrammica*) (GenBank Accession KC953095.1; Liu et al. 2014) to StrOccCau_1.0 using NCBI's BLAST+ version 2.4.0 tool BLASTN (Altschul et al. 1997; Camacho et al. 2009). We searched for long alignments to scaffolds with lengths not greatly exceeding 16,500 nt, the approximate size of the mitochondrial genomes of other owl (Aves: Strigiformes) species (Harrison et al. 2004; Liu et al. 2014; Mahmood et al. 2014; Hengjiu et al. 2016). We extracted the scaffold corresponding to the mitochondrial genome assembly using bioawk version 1.0 (Li 2013b) and annotated it using the MITOS WebServer version 806 (Bernt et al. 2013) (supplementary section 1.21, Supplementary Material online). We will refer to the mitochondrial and nuclear genome assemblies hereafter as StrOccCau_1.0_mito and StrOccCau_1.0_nuc, respectively.

Sex Identification

In order to determine the sex of the *S. o. caurina* individual that supplied the genetic sample for this genome assembly, we aligned nucleotide sequences of *S. varia* chromo-helicase-DNA binding protein-W (*CHD1W*) (GenBank Accession KF425687.1) and chromo-helicase-DNA binding protein-Z (*CHD1Z*) (GenBank Accession KF412792.1) to StrOccCau_1.0 using NCBI's BLAST+ version 2.4.0 tool BLASTN (Altschul et al. 1997; Camacho et al. 2009). We extracted the scaffolds that aligned to the *CHD1W* and *CHD1Z* sequences using bioawk version 1.0 (Li 2013b) and then used Geneious version 9.1.4 (Kearse et al. 2012; Biomatters 2016a) to predict the length of a PCR product resulting from amplification of this region with primers 2550 F and 2718 R (Fridolfsson and Ellegren 1999) (supplementary section 1.22, Supplementary Material online).

Repeat Annotation

We ran our genome through two separate series of repeat masking steps. The purpose of the first series was to produce

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a masked genome without masking of low complexity regions or simple repeats, which we could then use for downstream annotation steps. The purpose of the second series was to obtain an accurate assessment of the total repeat content of the genome, including low complexity regions and simple repeats. We first performed a homology-based repeat annotation of the genome assembly using RepeatMasker version 4.0.5 (Smit et al. 2013) and the repeat databases of the DFAM library version 1.3 (Wheeler et al. 2013) and the Repbase-derived RepeatMasker libraries version 20140131 (Jurka 1998, 2000; Jurka et al. 2005; Bao et al. 2015) without masking low complexity regions or simple repeats. We next performed a *de novo* modeling of the repeat elements in the genome using RepeatModeler version 1.0.8 (Smit and Hubley 2015) in order to create a database of repetitive regions in our genome assembly. We then further masked the genome by running RepeatMasker using the homology-based repeat-masked genome as input and the repeat database created by our RepeatModeler run and again not masking low complexity regions or simple repeats. The output was a twice-masked genome, hereafter "StrOccCau_1.0_masked." Finally, we repeated the above steps to perform a separate homology-based and *de novo* masking of the genome with RepeatMasker runs that included masking of low complexity regions and simple repeats in order to obtain an accurate estimate of the total repeat content of the genome (supplementary section 1.23, Supplementary Material online).

Gene Annotation

In order to annotate genes in the repeat-masked assembly, StrOccCau_1.0_masked, we followed the MAKER version 2.31.8 (Cantarel et al. 2008) pipeline as described in Campbell et al. (2014). As input for protein homology evidence, we provided MAKER the redundant protein set previously used to annotate 48 avian genomes (Zhang, Li C, et al. 2014). We used the genes found in our CEGMA run to train the gene prediction tool, Semi-HMM-based Nucleic Acid Parser or SNAP version 2006-07-28 (Korf 2004). As we independently performed repeat masking, we ran MAKER without further repeat masking. We combined all of the output gene annotations using the MAKER accessory scripts "fasta_merge" and "gff3_merge" (supplementary section 1.24, Supplementary Material online).

We assigned putative gene functions to the MAKER annotations by comparing the output MAKER protein fasta file to the Swiss-Prot UniProt release 2016_04 (Consortium 2015) database using NCBI's BLAST 2.2.31+ tool "blastp" (Altschul et al. 1997; Camacho et al. 2009). In order to identify proteins with known functional domains, we ran InterProScan version 5.18-57.0 (Jones et al. 2014) on the protein sequences generated by MAKER. We then filtered transcripts with an Annotation Edit Distance (AED) < 1 and/or a match to a domain in Pfam, a database of protein families (Finn et al. 2016), using the script "quality_filter.pl" supplied in MAKER version 3.00.0 (Cantarel et al. 2008). We compared the unfiltered and filtered GFF3 files by analyzing the AED values for all annotations using the script "AED_cdf_generator.pl" supplied in MAKER version 3.00.0 (Cantarel et al. 2008) and graphed the distribution of values using Matplotlib pyplot (Hunter 2007) (supplementary fig. S1, Supplementary Material online). Finally, we used GenomeTools version 1.5.1 (Gremme et al. 2013) to calculate annotation summary statistics, including distributions of gene lengths, exon lengths, number of exons per gene, coding DNA sequence (CDS) lengths (measured in amino acids), and intron lengths (supplementary section 1.24, Supplementary Material online) and graphed these using Matplotlib pyplot (Hunter 2007) (supplementary figs. S2–S6, Supplementary Material online).

Alignment

We aligned the filtered versions of all sequences from all libraries to StrOccCau_1.0_masked using the Burrows-Wheeler aligner, BWA-MEM version 0.7.12-r1044 (Li 2013a), and then merged, sorted, and marked duplicate reads using Picard version 1.104 (http://broadinstitute.github.io/picard; last accessed October 1, 2016). We then assessed the genome coverage, duplication level, and other statistics of each aligned sequence library using Picard version 1.141 (http://broadinstitute.github.io/picard; last accessed October 1, 2016) (supplementary section 1.25, Supplementary Material online). In order to obtain an estimate of the insert size of the mate pair libraries independent of the N-gaps in the scaffold sequences, we divided scaffolds into contigs at 25 or more N's, aligned the mate pair libraries to this set of contigs using BWA-MEM version 0.7.12-r1044 (Li 2013a), and then calculated insert sizes from these alignments (supplementary section 1.25, Supplementary Material online).

Microsatellite Analysis

We searched the repeat-masked and unmasked versions of our assembly for all microsatellite primers that have been designed from sequencing of the Mexican spotted owl (*S. o. lucida*) (Thode et al. 2002) as well as additional primers that were designed from sequences obtained from other strigid (Aves: Strigidae) species (Isaksson and Tegelström 2002; Hsu et al. 2003, 2006; Koopman et al. 2004; Proudfoot et al. 2005), but which have been used in population-level studies of *S. occidentalis* (Funk et al. 2008, 2010) and/or have been found to be useful in genetically determining F1 and F2 *S. occidentalis* $\times$ *S. varia* hybrids (Funk et al. 2007). We searched the assembly for 16 pairs of microsatellite primer sequences using NCBI's BLAST+ version 2.4.0 tool BLASTN (Altschul et al. 1997; Camacho et al. 2009) (supplementary section 1.26, Supplementary Material online).

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Barred Owl Divergence

In order to assess the genome-wide divergence of *S. occidentalis* and *S. varia*, we extracted genomic DNA from preserved tissue of a *S. varia* collected in Hamilton County, Ohio ([CNHM<USA-OH>:ORNITH:B41533]; hereafter referred to as "CMCB41533"; table 1) using a DNeasy Blood & Tissue Kit (Qiagen). We prepared a whole-genome library with an average insert length of 466 nt using a Nextera DNA Sample Preparation Kit (Illumina) and obtained 150 nt paired-end sequence data. We performed adapter and quality trimming of the sequence data using Trimmomatic version 0.32 (Bolger et al. 2014). We aligned the trimmed sequences to StrOccCau_1.0_masked using BWA-MEM version 0.7.12-r1044 (Li 2013a) and then merged the alignments, sorted the alignments, and marked duplicate sequences using Picard version 1.104 (http://broadinstitute.github.io/picard; last accessed October 1, 2016). We then calculated alignment statistics using Picard version 1.141 (http://broadinstitute.github.io/picard; last accessed October 1, 2016). We used Genome Analysis Toolkit (GATK) version 3.4-46 UnifiedGenotyper (McKenna et al. 2010; DePristo et al. 2011; Van der Auwera et al. 2013) to call variants using the *S. occidentalis* (Sequoia) and *S. varia* (CMCB41533) BWA-MEM-aligned, sorted, duplicate-marked bam files as simultaneous inputs (supplementary section 1.27, Supplementary Material online).

We then filtered the variants to exclude indels, sites of low genotyping quality, sites where the reference individual had a homozygous alternative allele genotype, and sites with coverage greater than the mean coverage plus five times the standard deviation, as suggested by the GATK documentation (https://software.broadinstitute.org/gatk/guide/article? id=3225; last accessed October 1, 2016). We used GNU cut version 8.21 (Ihnat et al. 2013) and GNU Awk (GAWK) version 4.0.1 (Free Software Foundation 2012) to calculate H_w, the mean number of nucleotide differences within *S. o. caurina* and *S. varia*, as well as H_b, the number of nucleotide differences between the two species, and then used these to estimate the fixation index (F_{ST}) (Hudson et al. 1992), a measure of population differentiation (supplementary section 1.27, Supplementary Material online). We then used an implementation of the pairwise sequentially Markovian coalescent model, PSMC version 0.6.5-r67 (Li and Durbin 2011; Li 2015), with 100 rounds of bootstrapping to estimate the effective population size (N_e) through time for both *S. o. caurina* and *S. varia* (supplementary section 1.28, Supplementary Material online).

Light-Associated Gene Analyses

We searched our *S. o. caurina* StrOccCau_1.0 assembly and the *T. alba* genome assembly (GenBank Accession GCA_000687205.1) for the presence of functional orthologs in nineteen genes that encode proteins with light-associated functions. These genes encode five visual pigment proteins (*LWS* [long wavelength-sensitive opsin], *SWS1* [short wavelength-sensitive 1 opsin], *SWS2* [short wavelength-sensitive 2 opsin], *Rh1* [rod opsin], *Rh2* [rod-like cone opsin]) (Davies et al. 2012); ten nonvisual photopigment proteins (*Opn3* [panopsin/encephalopsin], *Opn4m* [mammal-like melanopsin], *Opn4x* [*Xenopus*-like melanopsin], *Opn5* [neuropsin], *Opn5L1* [neuropsin-like 1], *Opn5L2* [neuropsin-like 2], *OpnP* [pinopsin], *RRH* [peropsin], *RGR* [retinal G protein-coupled receptor], *OpnVA* [vertebrate ancient opsin]) (Okano et al. 1994; Shen et al. 1994; Soni and Foster 1997; Sun et al. 1997; Blackshaw and Snyder 1999; Halford et al. 2001; Tarttelin et al. 2003; Bellingham et al. 2006; Tomonari et al. 2008); three enzymes involved in protection from UV radiation (*EEVS*-like, *MT-Ox*, *pOPC1* [photolyase]) (Kato et al. 1994; Osborn et al. 2015); and an enzyme involved in synthesizing red ketocarotenoid pigments (*CYP2J19* [carotenoid ketolase]) (Lopes et al. 2016; Mundy et al. 2016). We queried the genome assemblies of *S. o. caurina* and *T. alba* utilizing *in silico* probes that encompassed the exons, introns and 5′ and 3′ flanking sequences of the above genes (see supplementary table S3, Supplementary Material online for details on the probe sequences). We imported the *S. o. caurina* genome assembly into Geneious version 9.1.6 (Kearse et al. 2012; Biomatters 2016b) and used the included version of the NCBI BLAST+ BLASTn tool (Zhang et al. 2000) to search for the probes in our assembly. We used the web version of NCBI BLAST+ version 2.5.0 (Zhang et al. 2000) to align the probes against the *T. alba* genome assembly sequences in the NCBI Whole-Genome-Shotgun (WGS) contigs database. After recovering matches with our BLAST searches, we used the Geneious version 9.1.6 implementation of the MUSCLE aligner (Edgar 2004) to align the BLAST results to the probe sequences. We then used Geneious version 9.1.6 to manually adjust the alignments and examine the owl sequences for inactivating mutations, such as premature stop codons, frame shift indels (insertions/deletions), and splice site mutations. When BLAST searches were unsuccessful, we performed BLAST searches against the discarded < 1,000 nt contig set. In cases of further negative results, we used synteny data from Ensembl (version 86; Yates et al. 2016) to search for evidence of whole gene deletion (supplementary section 1.29, Supplementary Material online and supplementary table S3, Supplementary Material online). Specifically, we identified genes flanking the gene of interest in other vertebrate taxa with available contiguous genomic sequence through the relevant region, and used BLAST as noted earlier to align the reference sequences for these flanking genes to the genome assemblies of *S. o. caurina* and *T. alba*. If both flanking genes occurred on the same contig/scaffold and the intergenic sequence was not composed of missing data (N's), this provided evidence that the gene of interest had been deleted from the genome. In order to provide further evidence of gene deletion, we used the web version of NCBI BLAST+ version 2.5.0 blastn tool (Zhang et al. 2000) to align the assembly sequence

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intervening the flanking genes to available sequence data in the NCBI nucleotide database "nt" (Clark et al. 2016; NCBI Resource Coordinators 2016) to search for remnant sequences of untranslated gene regions.

In instances where we discovered evidence of potentially inactivating mutations in light-associated genes of one or both owl species, we performed dN/dS ratio (ω) analyses to test whether the owl orthologs displayed evidence of relaxation of the strength of natural selection. We obtained additional ortholog sequences for the following nonowl avian species using the web version of the NCBI BLAST+ version 2.5.0 blastn tool (Zhang et al. 2000) with the discontiguous megablast option to search the NCBI nucleotide database "nt" (Clark et al. 2016; NCBI Resource Coordinators 2016): *A. chrysaetos*, turkey vulture (*Cathartes aura*), speckled mousebird (*Colius striatus*), cuckoo roller (*Leptosomus discolor*), bar-tailed trogon (*Apaloderma vittatum*), rhinoceros hornbill (*Buceros rhinoceros*), downy woodpecker (*P. pubescens*), and the northern carmine bee-eater (*Merops nubicus*) (see supplementary table S9, Supplementary Material online for sequence information). After aligning the owl gene sequences with the outgroup taxa using MUSCLE (Edgar 2004) in Geneious version 9.1.6, we adjusted the alignments manually and removed all stop codons as well as any codon positions with questionable homology. We then modeled the evolution of the genes of interest using the codeml program from the PAML version 4.8 package (Yang 2007) assuming the Prum et al. (2015) phylogeny and two separate codon frequency models (F1X4 and F3X4). We created nested models and tested for statistically significant differences in model fits using likelihood ratio tests (parameters included model = 0 [one ratio] or 2 [nested models], fix_omega = 0, NSsites = 0, see supplementary tables S10 and S11, Supplementary Material online for additional information). Most models implemented branch tests, which assumed that ω differed across branches on the phylogeny, but was equal across a gene. We estimated the foreground ω on the *Tyto* branch for *OpnP*, the *Strix* and *Tyto* branches for *CYP2J19* and *Rh2*, and the crown (*Strix*+ *Tyto*) and stem Strigiformes branches for *Opn4m*. The background ω for each gene consisted of the remaining branches. In a few instances, we implemented branch-sites tests, which assumed differences in ω across the phylogeny while allowing for different ω values across different portions of a gene (parameters included model = 2, fix_omega = 1 [null] or 0 [alternative], omega = 1, NSsites = 2).

We additionally used the NCBI BLAST+ version 2.5.0 blastn tool (Zhang et al. 2000) with the discontiguous megablast option to align a reference *Opn4m* sequence to fifteen avian retinal transcriptomes, which included six owl species (Wu et al. 2016) in NCBI's Sequence Read Archive (SRA) (Leinonen et al. 2011; NCBI Resource Coordinators 2016) (see supplementary section 1.29, Supplementary Material online for additional transcriptome information). We imported the short reads that aligned into Geneious version 9.1.6 (Kearse et al. 2012; Biomatters 2016b) and mapped them to the reference sequence using the Geneious "map to reference" function and trying both the "medium sensitivity/fast" and "low sensitivity/fastest" settings.

Results

Contamination Assessment

Our search for nonvertebrate sequences in our assembly suggested that our assembly was only very minimally contaminated with nonvertebrate sequences. For only nine out of the 8,113 final assembly scaffolds, one of the five top alignments to the NCBI nucleotide database (Clark et al. 2016; NCBI Resource Coordinators 2016) was an alignment to a nonvertebrate sequence. Four of these scaffolds were short, ranging from 1,182 to 2,304 nt, and aligned to *Escherichia coli* sequence data. We removed these four scaffolds from the assembly. We kept the other five scaffolds in the assembly. The highest BLAST bit-score for scaffold-1085 was for an alignment to the telomere region of a human genome with 81% identity across 53% of the scaffold. The highest BLAST bit-scores for scaffold-1155 were for alignments to endogenous retrovirus regions of several vertebrate genomes. Three scaffolds (2014, 2160, and 3069) were longer scaffolds that aligned to vertebrate genome sequences with only small sequence portions that aligned to nonvertebrate sequence data; we did not feel this justified removing them from the assembly.

Mitochondrial Genome Identification

We identified scaffold-3674 as an assembly of the mitochondrial genome as it had a 14,649 nt alignment with 89.1% similarity to the *S. leptogrammica* mitochondrial genome. This length was the majority of the 21,628 nt scaffold-3674. After subtracting a block of 3,984 N's present in the scaffold, the length of scaffold-3674 is similar to that of other avian mitochondrial genomes (Mindell et al. 1997, 1998, 1999; Guan et al. 2016; Zhang et al. 2016). We were able to annotate all of the standard avian mitochondrial genes, except *ND6* and $tRNA^{Pro}$, which suggests that this assembly of the mitochondrial genome could be improved.

Genome Size

Our *k*-mer-based estimation with Preqc yielded an estimated genome length of 1.29 giga nucleotides (Gnt). This type of estimation generally underestimates the true genome size as it collapses *k*-mers from highly repetitive regions. The total length of all sequences in our gap-closed assembly was 1.88 Gnt, but this length included all singleton sequences (many of which were unassembled reads) and N-filled gaps. After removing all contigs and scaffolds <1,000 nt, the combined total length of all scaffolds was 1.26 Gnt.

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Assembly Statistics

Gap-closing improved the assembly continuity and completeness metrics (tables 3 and 4). Removing shorter length contigs/scaffolds improved the post gap closing assembly metrics at both the contig and the scaffold level. The unfiltered assembly had a scaffold N50 length of 1.836 Mnt and a contig N50 length of 81,400 nt. Removing contigs/scaffolds less than 300 nt increased the scaffold and contig N50 lengths over 2× to 3.916 Mnt and 168,721 nt, respectively, and generated the greatest relative increase in the other continuity metrics of any of the filtering options that we tried (supplementary table S4, Supplementary Material online). The highest scaffold and contig N50 lengths (3.983 Mnt and 171,882 nt, respectively) and the best other continuity metrics resulted from removing all contigs and scaffolds <1,000 nt, but this came at the slight expense of the completeness of the genome (supplementary table S4, Supplementary Material online; tables 3 and 4). Our gap-closed genome included complete sequences of 228 and at least partial sequences of 236 of the 248 CEGMA orthologs. We only lost one of these when we removed contigs and scaffolds <1,000 nt and retained 228 complete and 235 partial CEGMA orthologs in the filtered assembly (table 4). Except for the percentage of duplicated orthologs, which was notably higher as measured by the CEGMA analysis versus the BUSCO analysis, the results of the CEGMA and BUSCO analyses closely agreed. Both found at least partial sequences of over 90% of the conserved orthologs (235/248 = 94.76% CEGMA and 2,815/3,023 = 93.12% BUSCO orthologs) under scrutiny in the final assembly (table 4). Our final assembly contained 8,113 scaffolds and/or contigs with a scaffold N50 of 3.98 Mnt. The longest scaffold was 15.75 Mnt. The GC content was 41.31%. The N content was 1.10%.

The contig-level continuity statistics improved substantially when we allowed for longer blocks of intervening N's before demarcating separate contigs (supplementary table S4, Supplementary Material online). Relative to delineating contigs at every N (contig N50 of 51,301 nt), allowing up to 5 N's before demarcating a separate contig yielded an over 3× increase in the contig N50 of 155,200 nt. This was the greatest relative increase that we saw in the contig N50 length out of all the intervening N lengths that we tried, (supplementary table S4, Supplementary Material online). Allowing up to 25 N's before demarcating a separate contig resulted in the highest contig N50 (171.88 kilo nucleotides (knt); supplementary table S4, Supplementary Material online). In both continuity and completeness, our assembly compares favorably with those of the other avian genomes for which we calculated equivalent metrics (table 5).

Sex Identification

We determined from our assembly that the sequence came from the genome of a female *S. o. caurina*. The lengths of the *CHD1* markers on the sex chromosomes were 634 and 1,058 nt on scaffolds 806 and 4429, respectively. These lengths are in the size range of those amplified from *S. nebulosa* samples by previous researchers (600–650 and 1,200 nt for *CHD1Z* and *CHD1W*, respectively) (Fridolfsson and Ellegren 1999) and suggest that scaffolds 806 and 4429 are sequences from the Z and W chromosomes, respectively.

Repeat Annotation

The repeat annotation and masking of the genome examined 3,754,965 individual sequences totaling 1,882,109,172 nt. The homology-based repeat annotation resulted in GC content estimation of 44.15% and masked 21.02% of the assembly as repetitive. Repeat masking using a *de novo* model of the repeat elements estimated that an additional 0.55% of the assembly was repetitive. Due to the fact that some of the annotated repetitive elements overlapped, the following repeat category percentage values do not exactly sum to the 21.57% total genome repeat content. Interspersed repeat elements including retroelements, DNA elements (DNA transposons with no RNA intermediate), and unclassified elements comprised 9.31% of the assembly; of these, retroelements were the most common, constituting 8.96% of the assembly (table 6). Non-interspersed repeat elements including small RNA elements, satellites, simple repeats, and low complexity repeats comprised 12.33% of the assembly; of these, satellites were the most common, constituting 9.88% of the assembly.

Gene Annotation

The MAKER pipeline succeeded in annotating all contigs and scaffolds except one, scaffold-1363, which is 555,526 nt long and failed the annotation pipeline for an unknown reason. The MAKER pipeline's implementation of AUGUSTUS version 3.2.1 (Keller et al. 2011; Stanke 2015) predicted 19,692 proteins and transcripts *ab initio*. After quality filtering, we retained 16,718 annotated proteins and transcripts, 5,062 of which were non-overlapping *ab initio* predictions of proteins and transcripts.

Annotated gene sequence lengths ranged from 51 to 282,544 nt with a median length of 9,187.50 nt (supplementary fig. S2, Supplementary Material online). Coding sequence lengths varied from 51 to 66,303 nt with a median length of 1,137 nt (supplementary fig. S3, Supplementary Material online). Exon lengths extended to a maximum of 14,832 nt with a median length of 130 nt (supplementary fig. S4, Supplementary Material online). Intron lengths ranged from 45 to 57,529 nt with a median length of 910 nt (supplementary fig. S5, Supplementary Material online). The number of exons per gene ranged from 1 exon to 142 exons with a median number of six exons per gene (supplementary fig. S6, Supplementary Material online).

Alignment

The assembly contained 1,142, 612,682 nonN bases used in the calculation of the library alignment statistics. After all

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Table 3
Final Assembly Metrics

Assembly Version	No Gap-Closing, no Scaffolds, or Contigs Removed	Gap-Closed, No Scaffolds or Contigs Removed	Gap-Closed, Scaffolds and Contigs <1,000 nt Removed
Number of scaffolds	3,754,960	3,754,960	8,108
Total size of scaffolds	1,884,397,264 nt	1,882,081,621 nt	1,255,541,132 nt
Longest scaffold	15,783,852 nt	15,750,186 nt	15,750,186 nt
Shortest scaffold	128 nt	128 nt	1,000 nt
Number of scaffolds > 1 K nt	8,112 (0.2%)	8,095 (0.2%)	8,095 (99.8%)
Number of scaffolds > 10 K nt	1,754 (0.0%)	1,746 (0.0%)	1,746 (21.5%)
Number of scaffolds > 100 K nt	661 (0.0%)	661 (0.0%)	661 (8.2%)
Number of scaffolds > 1 M nt	303 (0.0%)	303 (0.0%)	303 (3.7%)
Number of scaffolds > 10 M nt	9 (0.0%)	9 (0.0%)	9 (0.1%)
Mean scaffold size	502 nt	501 nt	154,852 nt
Median scaffold size	150 nt	150 nt	1,904 nt
N50 scaffold length (L50 scaffold count)	1,843,286 nt (209)	1,836,279 nt (209)	3,983,020 nt (92)
N60 scaffold length (L60 scaffold count)	622,124 nt (370)	619,581 nt (371)	3,012,707 nt (129)
N70 scaffold length (L70 scaffold count)	255 nt (216,251)	255 nt (218,976)	2,162,240 nt (178)
N80 scaffold length (L80 scaffold count)	174 nt (1,110,583)	174 nt (1,113,245)	1,545,070 nt (246)
N90 scaffold length (L90 scaffold count)	143 nt (2,336,958)	143 nt (2,338,577)	618,731 nt (372)
scaffold %GC	42.81%	43.82%	41.31%
scaffold %N	2.89%	0.74%	1.10%
Percentage of assembly in scaffolded contigs	66.4%	65.7%	98.5%
Percentage of assembly in unscaffolded contigs	33.6%	34.3%	1.5%
Average number of contigs per scaffold	1.0	1.0	3.4
Average length of break (>25 Ns) between contigs in scaffold	311	703	716
Number of contigs	3,929,029	3,774,552	27,252
Number of contigs in scaffolds	179,939	22,372	21,478
Number of contigs not in scaffolds	3,749,090	3,752,180	5,774
Total size of contigs	1,830,109,624 nt	1,868,296,631 nt	1,241,823,123 nt
Longest contig	186,255 nt	1,259,046 nt	1,259,046 nt
Shortest contig	5 nt	128 nt	130 nt
Number of contigs > 1 K nt	123,891 (3.2%)	23,915 (0.6%)	23,915 (87.8%)
Number of contigs > 10 K nt	37,347 (1.0%)	12,373 (0.3%)	12,373 (45.4%)
Number of contigs > 100 K nt	58 (0.0%)	3,909 (0.1%)	3,909 (14.3%)
Number of contigs > 1 M nt	0 (0.0%)	8 (0.0%)	8 (0.0%)
Mean contig size	466 nt	495 nt	45,568 nt
Median contig size	150 nt	150 nt	6,702 nt
N50 contig length (L50 contig count)	7,855 nt (46,856)	81,400 nt (4,678)	171,882 nt (2,057)
N60 contig length (L60 contig count)	3,275 nt (81,600)	33,521 nt (8,121)	134,419 nt (2,876)
N70 contig length (L70 contig count)	254 nt (448,715)	255 nt (254,729)	98,604 nt (3,955)
N80 contig length (L80 contig count)	170 nt (1,346,255)	173 nt (1,148,692)	66,668 nt (5,484)
N90 contig length (L90 contig count)	142 nt (2,548,877)	142 nt (2,367,845)	34,559 nt (8,023)

NOTE.—Assembly (contaminant and mitochondrial sequences removed) metrics before gap-closing,after gap-closing,and after both gap-closing and removal of all contigs and scaffolds <1,000 nt in length. Strings of 25 or more N's broke scaffolds into contigs.

filters, the total mean coverage for the paired and unpaired data from all of the sequenced libraries aligned to the repeat-masked genome was 60.43×. The MP11 kb mate-pair library had the highest proportion of duplicate bases (60.1%) and the PCR-free library noPCR550 bp had the lowest (0.3%) (table 7).

Insert sizes of mate pair libraries determined by mapping quality-filtered reads back to the genome assembly gave lower inserts than were expected based on bioanalyzer traces. Whereas the bioanalyzer traces gave evidence that the MP4, MP7, and MP11 kb libraries had insert lengths of ∼4.2, 7.1, and 10.7 knt, respectively, the results from mapping to the whole genome assembly suggested that the insert lengths were instead 3.3, 5.9, and 9.6 knt, respectively. We hypothesized that this difference may have been due to the number of N's added during scaffolding, we also mapped the sequences from these libraries to the assembly with all scaffolds decomposed into their constituent contigs. This yielded

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Table 4
Summary of Conserved Ortholog Searches

Assembly	Draft. No Gap-Closing, Contigs/Scaffolds < 300 nt Removed	Draft. Gap-Closed, No Removal of Small Contigs/Scaffolds	Final. Gap-Closed, Contigs/Scaffolds <1,000 nt Removed	Final. Gap-Closed, Contigs/Scaffolds <1,000 nt Removed
Method	CEGMA	CEGMA	CEGMA	BUSCO
Total conserved orthologs examined	248	248	248	3,023
Complete orthologs (% of total)	221 (89.11%)	228 (91.94%)	228 (91.94%)	2,605 (86.17%)
At least partial orthologs (% of total)	235 (94.76%)	236 (95.16%)	235 (94.76%)	2,815 (93.12%)
Duplicated orthologs (% of total)	92 (37.10%)	83 (33.47%)	99 (39.92%)	46 (1.52%)
Missing orthologs	13 (5.24%)	12 (4.84%)	13 (5.24)%	208 (6.88%)

Note.—Comparison of the number of conserved orthologous genes found in the final assembly (gap-closed,contigs/scaffolds <1,000 nt removed) using the CEGMA and BUSCO tools. In order to illustrate the effect of gap-closing and removal of small fragments on assembly completeness metrics,also included are the results of CEGMA gene searches conducted on two draft versions of the final assembly where we either did not perform gap-closing and removed contigs/scaffolds < 300 nt or performed gap-closing and did not remove any small contigs/scaffolds.

Table 5
Comparative Statistics of Avian Genomes

Species	Common name	Scaffold N50 (nt)	No. Scaffolds/ Contigs	Contig N50 (nt)	Length (Gnt)	Ns (%)	Complete CEGs (% of 248)	Partial CEGs (% of 248)
S. o. caurina	Northern Spotted Owl	3,983,020	8,108	171,882	1.26	1.10	228 (91.94%)	235 (94.76%)
T. alba	Barn Owl	51,873	166,092	19,113	1.14	1.02	144 (58.06%)	198 (79.84%)
P. pubescens	Downy Woodpecker	2,086,781	85,828	29,578	1.17	3.72	196 (79.03%)	216 (87.10%)
T. guttata	Zebra Finch	62,374,962	37,095	38,644	1.23	0.75	192 (77.42%)	214 (86.29%)
H. leucocephalus	Bald Eagle	669,725	346,419	10,218	1.26	3.97	217 (87.50%)	240 (96.77%)
A. chrysaetos	Golden Eagle	9,230,743	1,141	215,151	1.19	1.07	226 (91.13%)	238 (95.97%)
C. pelagica	Chimney swift	3,839,435	60,234	33,918	1.13	4.02	191 (77.02%)	222 (89.52%)
G. gallus	Chicken	82,310,166	23,474	2,905,620	1.23	0.96	226 (91.13%)	237 (95.56%)

Note.—Comparative statistics of our *S. o. caurina* assembly with those of a selection of other avian genome assemblies.

average insert sizes of 3.3, 6.0, and 10.0 knt, which suggest some potential for improving N gap lengths, but that the N stretches in the scaffolds are good approximations of the lengths of missing, intervening sequences.

Microsatellite Analysis

We found 15 out of the 16 pairs of microsatellite primers for which we searched in the genome assembly (table 8). We found loci 4E10, 4E10.2, and Oe149 on scaffold-11. The distance from the forward 4E10.2 primer to the forward 4E10 primer is 12,172 nt in our assembly, which confirms the characterization of the loci 4E10 and 4E10.2 as linked within 40 kb by the original authors who described these loci using sequences obtained from the same cosmid (Thode et al. 2002). The reverse 4E10 primer is 717,153 nt distant from the forward Oe149 primer. The remaining primer pairs aligned to separate assembly scaffolds (table 8).

Barred Owl Divergence

We estimated the nuclear genome-wide nucleotide diversity (H_W) of *S. o. caurina* as 2.008×10^{-4} and that of *S. varia* as 2.352×10^{-3}. We estimated the genome-wide nucleotide diversity between *S. o. caurina* and *S. varia* (H_b) as 7.042×10^{-3} and calculated an F_{ST} of 0.819.

PSMC Analysis

Our pairwise sequentially Markovian coalescent (PSMC) model analyses suggested that the N_e of both *S. o. caurina* and *S. varia* was substantially higher in the past and has been in decline since ~100,000 or 80,000 years before present, respectively (fig. 1). The estimated peak N_e of *S. o. caurina* was more than an order of magnitude lower than that of *S. varia* (~20,000 and 250,000 for *S. o. caurina* and *S. varia*, respectively). The most recent estimate that the PSMC analysis provided for the N_e of *S. o. caurina* was also more than an order of magnitude lower than that of *S. varia* (~4,000 and 50,000 for *S. o. caurina* and *S. varia*, respectively).

Light-Associated Gene Analyses

Seven of the nineteen genes encoding proteins with light-associated functions that we examined displayed evidence of inactivation or whole gene deletion in one or both owl species (supplementary table S3, Supplementary Material online; Hanna et al. 2017). We found no BLAST alignments of

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Table 6
Repetitive Element Summary

Type Level 1	Type Level 2	Type Level 3	Type Level 4	Number of Elements	Element Total Length (nt)	Assembly Portion (%)
Total interspersed repeats					175,287,790	9.31
	Total retroelements			727,006	168,672,903	8.96
	Retroelement	SINE		40,360	4,770,020	0.25
	Retroelement	SINE	ALU	53	6,194	0.00
	Retroelement	SINE	MIR	15,510	1,558,420	0.08
	Retroelement	Penelope		169	35,110	0.00
	Retroelement	Total LINEs		486,310	115,604,290	6.14
	Retroelement	LINE	LINE1	622	58,117	0.00
	Retroelement	LINE	LINE2	3,116	317,864	0.02
	Retroelement	LINE	L3/CR1	28,122	5,153,289	0.27
	Retroelement	LINE	CRE/SLACS	0	0	0.00
	Retroelement	LINE	L2/CR1/Rex	452,030	109,807,316	5.83
	Retroelement	LINE	R1/LOA/Jockey	0	0	0.00
	Retroelement	LINE	R2/R4/NeSL	131	44,590	0.00
	Retroelement	LINE	RTE/Bov-B	15	3,492	0.00
	Retroelement	LINE	L1/CIN4	98	23,441	0.00
	Retroelement	Total LTR elements		200,336	48,298,593	2.57
	Retroelement	LTR	BEL/Pao	0	0	0.00
	Retroelement	LTR	ERV_classI	983	122,219	0.01
	Retroelement	LTR	ERV_classII	400	54,854	0.00
	Retroelement	LTR	ERVL	436	91,660	0.00
	Retroelement	LTR	ERVL-MaLRs	51	4,838	0.00
	Retroelement	LTR	Gypsy/DIRS1	111	14,921	0.00
	Retroelement	LTR	Retroviral	197,967	47,947,799	2.55
	Retroelement	LTR	Ty1/Copia	0	0	0.00
	Total DNA elements			37,526	5,628,486	0.30
	DNA element		En-Spm	0	0	0.00
	DNA element		hAT-Charlie	418	28,220	0.00
	DNA element		hobo-Activator	4,235	719,417	0.04
	DNA element		MuDR-IS905	0	0	0.00
	DNA element		PiggyBac	0	0	0.00
	DNA element		Tc1-IS630-Pogo	806	141,663	0.01
	DNA element		TcMar-Tigger	528	39,074	0.00
	DNA element		Tourist/Harbinger	9,255	958,360	0.05
	DNA element		Other (Mirage, P-element,Transib)	0	0	0.00
	Rolling-circles			0	0	0.00
	Unclassified interspersed repeats			6,225	986,401	0.05
Total noninterspersed repeats				1,907,394	232,038,709	12.33
	Small RNA			12,051	1,645,166	0.09
	Satellites			1,261,021	185,995,538	9.88
	Simple repeats			564,508	40,568,395	2.16
	Low complexity repeats			69,814	3,829,610	0.20

Note.—Summary of the repeat elements found during two rounds of repeat masking (homology-based followed by denovo-model-based masking). Depending on the type of repeat element, we provide information at different category summary levels. We use the "Type level" column headings to organize these categories.

SWS1 to either the *S. o. caurina* or the *T. alba* assembly. However, the genes flanking *SWS1* in zebra finch (*T. guttata*) and human (*Homo sapiens*), *FLNC* (Filamin-C) and *CALU* (Calumenin) (Ensembl version 86; Yates et al. 2016), are both present in the *S. o. caurina* genome assembly, but they are located on different scaffolds. Without increased genomic continuity, it is difficult to discern whether chromosomal rearrangement has occurred or whether this is a case of simple gene deletion. Recent searches in crocodilian (Crocodilia) genomes similarly found *FLNC* and *CALU* on separate contigs with *SWS1* missing from the assemblies (Emerling 2017a), which suggests that this may be a problematic region to assemble. NCBI's Eukaryotic Genome Annotation (EGA) pipeline did not find *FLNC* and *CALU* in

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Table 7
Library Alignment Statistics

Library	Mean Paired and Unpaired Read Genome Coverage Postfiltering (X)	SD of Paired and Unpaired Read Genome Coverage Postfiltering (X)	Fraction of Aligned Bases From Unpaired Reads	Total Fraction of Filtered Aligned Bases	Fraction Aligned Bases Filtered Due to Mapping Quality < 20	Fraction Aligned Bases Filtered as Duplicates	Fraction Aligned Bases Filtered as Low Quality With Q<20	Fraction Aligned Bases Filtered as Second Observation From Overlapping Reads	Fraction Aligned Bases Filtered From Regions Already with > 1,000× coverage
Nextera350bp lane 1	4.369	5.484	0.048	0.533	0.060	0.444	0.004	0.023	1.52E-03
Nextera350bp lane 2	11.162	8.960	0.039	0.559	0.056	0.480	0.005	0.017	1.43E-03
Hydroshear	1.093	2.784	0.004	0.549	0.033	0.429	0.005	0.081	2.03E-03
Nextera550bp lane 1	2.741	3.708	0.393	0.096	0.034	0.038	0.011	0.011	1.05E-03
Nextera550bp lane 2	5.790	5.435	0.327	0.126	0.032	0.066	0.019	0.008	1.26E-03
Nextera700bp	23.357	14.710	0.041	0.216	0.046	0.126	0.009	0.032	3.64E-03
noPCR550bp	3.244	2.661	0.241	0.059	0.013	0.003	0.014	0.029	4.32E-04
PCR900bp	1.978	1.894	0.073	0.052	0.012	0.024	0.014	0.001	3.34E-04
MP4kb	2.528	2.745	0.300	0.361	0.048	0.306	0.002	0.004	5.36E-04
MP7kb	2.528	2.734	0.256	0.449	0.045	0.397	0.002	0.004	4.53E-04
MP11kb	1.641	2.205	0.168	0.652	0.046	0.601	0.001	0.004	2.56E-04
CMCB41533	15.552	12.253	0.030	0.341	0.299	0.037	2.37E-04	2.59E-03	2.50E-03

Note.—Alignment statistics for all Sequoia (*Strix occidentalis caurina*) libraries and the CMCB41533 (*Strix varia*) library calculated using Picard's CollectWgsMetrics.

the *T. alba* genome assembly (NCBI *T. alba* Annotation Release 100; NCBI Accession GCF_000687205.1), but the absence of these genes in the assembly may be due to low assembly quality (Zhang, Li B, Li C, et al. 2014).

SWS2 and *LWS* are adjacent on the same chromosome in the Carolina anole (*Anolis carolinensis*) and African clawed frog (*Xenopus laevis*) genome assemblies and are flanked by *MECP2* (methyl-CpG binding protein 2) in *A. carolinensis* and *X. laevis*, *AVPR2* (arginine vasopressin receptor 2) in *X. laevis*, and *TEX28* (testis expressed 28) in *A. carolinensis* (Ensembl version 86; Yates et al. 2016). We did not obtain BLAST alignments to *SWS2* or *LWS* for the *T. alba* assembly and NCBI's EGA pipeline did not find *MECP2*, *AVPR2*, or *TEX28* (NCBI *T. alba* Annotation Release 100; NCBI Accession GCF_000687205.1), which suggests that this portion of the genome, like the *SWS1* region, may be challenging to assemble. Although we found *SWS2* and *LWS* in our *S. o. caurina* assembly, we only obtained partial coding sequences with elevated GC content of 66.9% and 68.0%, respectively. Our *S. o. caurina* assembly contained a partial *SWS2* exon 1 sequence as well as complete exon 2 and 3 sequences with all three exons found on two separate scaffolds (scaffolds 4153 and 7110). The sequences of these exons on the two scaffolds were 100% identical except for one difference in exon 3. Given the high sequence similarity and the recovery of the same portions of the *SWS2* coding region, these duplicate sequences are likely an artifact of the assembly process and do not indicate gene duplication.

SWS2, *LWS*, *Rh1*, and *Rh2* in *S. o. caurina* and *Rh1* in *T. alba* showed no evidence of potentially inactivating mutations. However, *Rh2* in *T. alba* displayed a 29 nt deletion in exon 1, single premature stop codons in both exons 2 and 3, and a 2 nt deletion in exon 4. Our modeling of the sequence evolution of *Rh2* in *S. o. caurina* and *T. alba* yielded evidence that selection has become relaxed in *T. alba* ($\omega = 0.22$–0.37; $P < 0.00001$) relative to other avian taxa ($\omega = 0.03$–0.06), which is consistent with pseudogenization of this gene. A branch test of *S. o. caurina* also displayed evidence of relaxed selection on *Rh2* with an elevated ω (0.16–0.21; $P < 0.05$) relative to the background. Our branch-sites test evaluated whether there was indication of positive selection across a subset of sites, but it did not yield any evidence that the elevated ω was due to adaptive evolution. We did find nine missense mutations in *S. o. caurina* that were not found in any of the non-owl avian species, but none of these were at known conserved sites (Carleton et al. 2005), which suggests that they have not resulted in a loss of function.

We were unable to recover *OpnP* in our *S. o. caurina* assembly, but together on the same scaffold we found the genes that flank *OpnP* in the chicken (*G. gallus*) and the collared flycatcher (*Ficedula albicollis*) genome assemblies, *TEX14* (testis expressed sequence 14) in *G. gallus* and *DOC2B* (double C2 domain beta) in *G. gallus* and *F. albicollis* (Ensembl version 86; Yates et al. 2016). Our BLAST of the sequence

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Table 8
Genomic Locations of Selected Microsatellite Loci

Locus	Primer	References	Usage Comments	Length Primer	Length Alignment	Mismatches	Genome Scaffold	Genome Start	Genome End	Microsatellite Length (nt)
13D8	F	(Thode et al. 2002)	population genetics (Funk et al. 2008, 2010)	22	22	0	scaffold88	4,241,040	4,241,019	187
13D8	R			21	21	0	scaffold88	4,240,854	4,240,874	
15A6	F	(Thode et al. 2002)	population genetics (Funk et al. 2008, 2010)	21	21	0	scaffold233	2,208,703	2,208,723	148
15A6	R			19	16	0	scaffold233	2,208,847	2,208,832	
1C6	F	(Thode et al. 2002)	None	20	20	0	scaffold178	2,550,734	2,550,753	110
1C6	R			20	20	0	scaffold178	2,550,843	2,550,824	
4E10	F	(Thode et al. 2002)	None	22	22	0	scaffold11	768,391	768,371	230
4E10	R			22	22	0	scaffold11	768,162	768,183	
4E10.2	F	(Thode et al. 2002)	population genetics (Funk et al. 2008, 2010)	18	18	0	scaffold11	780,562	780,579	226
4E10.2	R			18	18	0	scaffold11	780,787	780,770	
6H8	F	(Thode et al. 2002)	population genetics (Funk et al. 2008, 2010)	21	21	0	scaffold103	3,773,885	3,773,865	93
6H8	R			16	16	0	scaffold103	3,773,793	3,773,808	
8G11	F	(Thode et al. 2002)	None	18	—	—	—	—	—	—
8G11	R			17	—	—	—	—	—	
Bb126	F	(Isaksson & Tegelström 2002)	hybrid diagnostic (Funk et al. 2007)	20	20	0	scaffold219	2,548,147	2,548,166	185
Bb126	R			24	24	0	scaffold219	2,548,331	2,548,308	
BOOW18	F	(Koopman et al. 2004)	hybrid diagnostic (Funk et al. 2007)	19	19	1	scaffold244	648,444	648,426	205
BOOW18	R			20	20	1	scaffold244	648,240	648,259	
FEPO5	F	(Proudfoot et al. 2005)	population genetics (Funk et al. 2008, 2010)	22	22	0	scaffold138	720,315	720,336	270
FEPO5	R			25	25	2	scaffold138	720,584	720,560	
Oe045	F	(Hsu et al. 2003)	hybrid diagnostic (Funk et al. 2007)	23	23	2	scaffold173	3,777,655	3,777,677	127
Oe045	R			19	19	0	scaffold173	3,777,781	3,777,763	
Oe053	F	(Hsu et al. 2003)	population genetics (Funk et al. 2008, 2010)	23	23	1	scaffold136	299,240	299,262	218
Oe053	R			22	22	1	scaffold136	299,457	299,436	
Oe128	F	(Hsu et al. 2003)	hybrid diagnostic (Funk et al. 2007),population genetics (Funk et al. 2008, 2010)	27	27	0	scaffold722	802,232	802,206	319
Oe128	R			24	24	0	scaffold722	801,914	801,937	
Oe129	F	(Hsu et al. 2006)	population genetics (Funk et al. 2008, 2010)	24	21	2	scaffold529	3,066,759	3,066,739	266

(continued)

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Table 8 Continued

Locus	Primer	References	Usage Comments	Length Primer	Length Alignment	Mismatches	Genome Scaffold	Genome Start	Genome End	Microsatellite Length (nt)
Oe129	R			24	24	1	scaffold529	3,066,497	3,066,520	
Oe149	F	(Hsu et al. 2006)	population genetics (Funk et al. 2008, 2010)	21	21	1	scaffold11	51,010	50,990	258
Oe149	R			20	20	0	scaffold11	50,753	50,772	
Oe3-7	F	(Hsu et al. 2003)	population genetics (Funk et al. 2008, 2010)	20	19	1	scaffold35	572,329	572,347	129
Oe3-7	R			23	23	0	scaffold35	572,456	572,434	

Note.—Locations of commonly used microsatellite loci in our draft genome assembly. We searched for all of the primer pairs used in several *S. occidentalis* population genetics studies as well all of those designed for use in *S. o. lucida* (Thode et al. 2002). The "Primer" column designates the forward or reverse primer with "F" or "R," respectively. The "Reference" column gives the citation of the publication that originally described each primer pair. The "Comment" column gives the citation(s) of the publication(s) in which a primer pair has been used for population-level study of *S. occidentalis* or and/or study of *S. occidentalis* x *S. varia* hybrids. "Length alignment" refers to the length of the BLASTN (Altschul et al. 1997; Camacho et al. 2009) alignment. The "Microsatellite length" refers to the inferred length of the microsatellite PCR product based on the length of the primers and their mapping positions in the genome assembly.

intervening *TEX14* and *DOC2B* in our *S. o. caurina* assembly revealed similarity (8% query coverage, 82% identity) with the 5′ untranslated region of *G. gallus OpnP*. Together, these provide strong evidence of whole gene deletion of *OpnP* in *S. o. caurina*. *OpnP* in *T. alba* is a pseudogene with numerous inactivating mutations, including the following: a start codon mutation (ACA), 13 nt deletion, 2 nt insertion, and 1 nt deletion in exon 1, a 1 nt deletion in exon 2, a 21 nt deletion of the intron 3-exon 4 boundary, a 7 nt deletion and 2 nt deletion in exon 4, and a 1 nt deletion in exon 5. We assembled sequences from outgroup taxa and confirmed that these mutations are unique to *T. alba*. Our dN/dS ratio analyses strongly suggested relaxed selection on the *T. alba* branch ($\omega = 0.51$–0.7; $P < 0.00001$) compared with purifying selection on the background branches ($\omega = 0.11$–0.18).

Opn4m displays evidence of inactivation in both *S. o. caurina* and *T. alba*, with both species sharing a 4 nt deletion in exon 8. Additionally, *S. o. caurina* has a premature stop codon in exon 8 and *T. alba* possesses a splice donor mutation (GT to AT) in intron 11. Comparisons with outgroup taxa confirmed that these mutations were unique to owls, but also demonstrated that other bird species have putative inactivating mutations in this gene, including the golden eagle (*A. chrysaetos*) with a premature stop codon in exon 9; speckled mousebird (*C. striatus*) with a 1 nt deletion in exon 9, splice donor mutation in intron 9 (GT to TT), and premature stop codon exon 11; cuckoo roller (*L. discolor*) with a splice donor mutation in intron 10 (GT to GA); and rhinoceros hornbill (*B. rhinoceros*) with a start codon mutation (ATG to CTG). We performed dN/dS ratio analyses after removing all exons that contained putative inactivating mutations. The results indicated that the average ω for the crown owl branches is elevated ($\omega = 0.45$; $P < 0.01$) relative to the background ($\omega = 0.19$), which does not meet the expectation of neutral evolution predicted if the shared 4 nt deletion led to a loss of function of *Opn4m*. Branch-sites tests yielded evidence of positive selection on some portions of the gene for both owl branches, but this signal was not a significantly better fit than the null. Our BLAST of an *Opn4m* sequence to fifteen bird retinal mRNA short read databases, which included data from six owl species, yielded alignments to all fifteen transcriptomes. Further investigation of these sequences in Geneious revealed evidence of different isoforms of *Opn4m*. When we used lower sensitivity alignment settings, the assemblies of mapped sequences generally terminated after exon 8 (the exon with the 4 nt deletion), suggesting that this is an abundant transcript isoform. However, using higher sensitivity alignment settings generated assemblies of multiple transcripts with distinct sequences at some of the exon–intron boundaries.

Finally, *CYP2J19* displays evidence of inactivation in both owl species. *S. o. caurina* has a 1 nt insertion and 2 nt deletion in exon 9. As Emerling (2017c) described, the *T. alba* assembly contains a premature stop codon in each of exons 1, 5, and 6 as well as a 5 nt deletion in exon 3. Both the *S. o. caurina*

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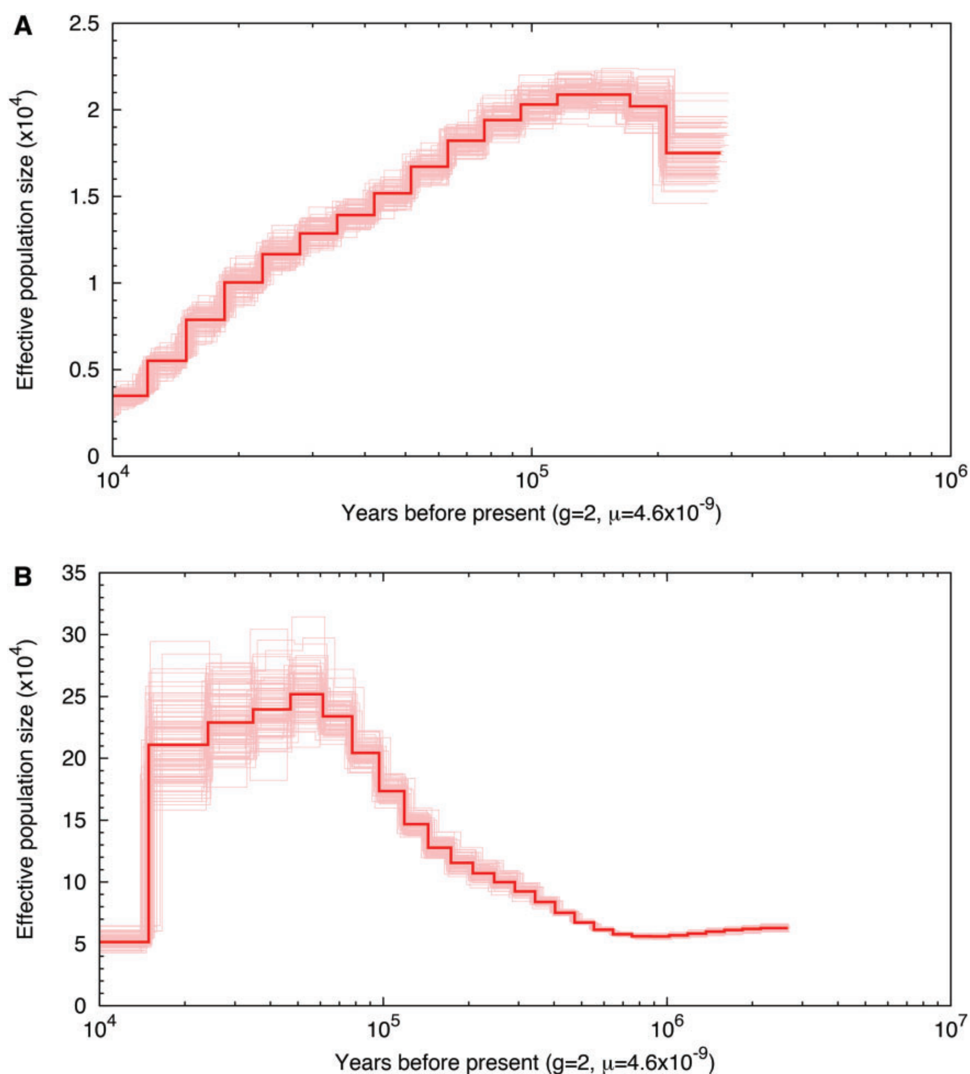

Fig. 1—Demographic history of *Strix occidentalis caurina* and *Strix varia* with bootstrap replicates. (Panel A) depicts the demographic history estimated for *S. o. caurina*. (Panel B) depicts the demographic history estimated for *Strix varia*.

($\omega = 0.33$–0.34; $P < 0.05$) and *T. alba* ($\omega = 0.68$–0.72; $P < 0.0001$) branches have elevated dN/dS ratios compared with the background (0.15–0.16), which is consistent with the hypothesis that these mutations have led to a loss of function of *CYP2J19*.

Discussion

Genome Characterization

Direct comparison of assembly metrics between our *S. o. caurina* assembly and seven other avian genome assemblies, including the avian model organisms chicken (*G. gallus*) and zebra finch (*T. guttata*), revealed that the *S. o. caurina* assembly is in the top tier of genomes in both continuity and completeness (table 5). Only the golden eagle (*A. chrysaetos*), zebra finch, and chicken genomes had better continuity statistics as measured by scaffold and contig N50s. We compared the relative completeness of the assemblies by searching for a set of 248 CEGs using CEGMA. Of the assemblies that we compared, we found the highest number of complete conserved gene sequences in our *S. o. caurina* assembly (228 complete CEGs), surprisingly surpassing even the chicken genome (226 complete CEGs). In terms of at least partially complete sequences of conserved genes, our *S. o. caurina* assembly contained only two fewer than the chicken genome (235 vs. 237 partial CEGs). Our assembly is both more complete and more contiguous than that of *T. alba*, the only other owl assembly currently available (*S. o. caurina* vs. *T. alba* assembly statistics include 235 vs. 198 CEGs at least partially present, scaffold N50 of $\sim 4.0 \times 10^6$ nucleotides vs. $\sim 5.2 \times 10^4$ nucleotides, and contig N50 of $\sim 1.7 \times 10^5$ nucleotides vs. $\sim 1.9 \times 10^4$ nucleotides).

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The number of annotated genes and the percentage of interspersed repeat elements in our *S. o. caurina* assembly are similar to those seen in other avian genomes (Zhang, Li B, Li C, et al. 2014). The number of annotated genes in our assembly (16,718 genes) was very similar to the number in the high-quality chicken and zebra finch genomes (16,516 and 17,471 genes, respectively) (Zhang, Li B, Li C, et al. 2014). These values were at the upper end of the range seen in the analysis of the gene annotations of 48 avian genomes (13,454–17,471 genes) (Zhang, Li B, Li C, et al. 2014). Similar to the number of annotated genes, the percentage of interspersed repeat elements in our *S. o. caurina* assembly (9.31%) closely matched the percentage found in the chicken and zebra finch genomes (9.82% and 9.68%, respectively) (Zhang, Li B, Li C, et al. 2014). These values fell at the higher end of the range seen in the analysis of 48 avian genomes (4.11–9.82%) if one excludes the downy woodpecker (*P. pubescens*) outlier (22.15%) (Zhang, Li B, Li C, et al. 2014).

Our searches for CEGs with both our CEGMA and BUSCO analyses revealed that our *S. o. caurina* assembly lacks only 5–7% of conserved orthologs, which is similar to the 4.4% we observed to be absent in the assembly of the chicken genome. Genome size data estimated from flow cytometry measurement of red blood cells exist for two *S. occidentalis* congeners. The nuclear genome lengths of the tawny owl (*Strix aluco*) and the great gray owl (*S. nebulosa*) are ~1.56 Gnt (De Vita et al. 1994; Doležel et al. 2003) and 1.61 Gnt (Doležel et al. 2003; Vinogradov 2005), respectively, which average to 1.59 Gnt. As compared with this average, the shorter total length of our scaffolded *S. o. caurina* assembly (~1.26 Gnt) suggests that 21% of the full genome sequence length of *S. o. caurina* remains unrepresented in this assembly. This is similar to the ~17.8% unrepresented sequence in the 1.19 Gnt golden eagle genome, assuming a genome size of ~1.45 Gnt (Doležel et al. 2003; Nakamura et al. 1990). The unrepresented sequence may consist largely of difficult-to-assemble repetitive content (Wicker et al. 2005; Yamada et al. 2004). These data illustrate that the *S. o caurina* assembly is comparable to the top tier of avian genomes assembled to date, but, as with all avian genomes, there is still improvement to be made.

Previous work on *Strix* karyotypes suggests that *S. occidentalis* likely has a typical avian karyotype of 2n = 80–82 (Renzoni and Vegni-Talluri 1966; Hammar 1970; Belterman and Boer 1984; Rebholz et al. 1993;). Assuming 1n = 41 chromosomes, the 8,100 scaffolds in our assembly yield ~198 scaffolds per chromosome. However, this number may not be a very meaningful estimate of the number of sequence blocks per chromosome as *Strix* shares with other birds the feature of possessing chromosomes in a wide range of sizes with the majority of the karyotype (~35 of the 41 chromosomes) comprised of microchromosomes and just 6 macrochromosomes (Rebholz et al. 1993).

The SOAPdenovo2 version 2.04 (Luo et al. 2012) assembler does not remove short sequences, which were mostly unincorporated reads. We removed all contigs and scaffolds <1,000 nt for our final assembly and used the resulting assembly in downstream analyses. We felt that removal of these small sequences was warranted as sequences shorter than 1,000 nt are unlikely to be useful in assessing synteny or gene structure. Some commonly used assemblers, such as ALLPATHS-LG, do not output contigs/scaffolds <1,000 nt (Gnerre et al. 2011). Indeed, the authors of the ALLPATHS-LG description removed contigs/scaffolds <1,000 nt in the comparisons of their assembler's functionality with other genome assemblers (Gnerre et al. 2011). Removal of these short sequences post assembly allowed us to better compare across assemblies and to effectively analyze what was actually assembled.

Our CEGMA results suggest that we lost minimal genome information (only 1 out of 248 conserved orthologs examined) by removing assembly contigs/scaffolds <1,000 nt. This validated our decision to remove these short sequences and confirmed that it was likely not worth the increase in processing time to retain these small genome fragments in downstream analyses. Additionally, larger genome assembly fragments have greater structural information.

In order to calculate the contig N50 statistic, scaffolds must be decomposed into constituent contigs. We explored how the criteria for splitting scaffolds into contigs affected assembly statistics. As one might expect, allowing longer blocks of N's before breaking a scaffold into contigs resulted in better continuity statistic values. We chose to allow up to 25 N's before separating contigs in our final assembly metric calculations as this was the default used in the "assemblathon_stats.pl" script used for calculating assembly statistics of the Assemblathon 2 genome assemblies (Bradnam et al. 2013). Indeed, even though the "assemblathon_stats.pl" script allowed the user to set a flag to change the number of N's that would separate contigs, our examination of the code revealed that the 25 N's was actually hard-coded into the script and overrode any value set by the user.

We found that our assemblies had better continuity metrics when we did not include all of our available short read data in the assembly. Of particular benefit was the exclusion of the Hydroshear data set, which displayed a high level of sequence duplication. This suggests that checking libraries for evidence of elevated levels of duplication prior to an assembly could be beneficial.

We found that all of the microsatellite primer pairs previously used for *S. occidentalis* genetic studies (Funk et al. 2007, 2008, 2010) mapped at reasonable distances from each other and predicted PCR products in normal microsatellite size ranges. We found no evidence of linkage except for three primer pairs that mapped to the same scaffold. The other 11 primer sets that we were able to align to the assembly

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mapped to separate scaffolds. A chromosome-level genomic sequence assembly would help further evaluate the independence of these loci.

Genome-Wide Divergence of Spotted Owl and Barred Owl

As *S. o. caurina* and *S. varia* are separate species, we expected a high genome-wide F_{ST} estimate, but our estimate is elevated even relative to values calculated for other congeneric bird species pairs (Toews et al. 2016). It is difficult to interpret this value; however, as the genome-wide nucleotide diversity within *S. varia* is ∼10-fold greater than that of *S. o. caurina*. We hypothesize that a difference in N_e for the two species is likely the largest contributor to this difference in nucleotide diversity, especially as the Marin *S. o. caurina* population of which our *S. o. caurina* genome is a sample is known to be an isolated population of this extinction-threatened species (Barrowclough et al. 2005). Following from the 10-fold difference in nucleotide diversity of the two species' genomes, our PSMC analyses suggested that the N_e of *S. varia* was consistently approximately an order of magnitude greater than that of *S. o. caurina* over the past 100,000 years. The PSMC analyses also suggested that the N_e of both *S. o. caurina* and *S. varia* has been in decline over the past tens of millennia, but we caution that precise timing of the past maximum N_e for both species and its subsequent decline is highly dependent on the values chosen for the substitution rate and generation time, which likely require further optimization for these *Strix* species and for owls in general.

Light-Associated Gene Analyses

We have provided genomic evidence of inactivation and deletion of genes with light-associated functions in two species of predominantly nocturnal owls. Ancestral birds likely possessed tetrachromatic color vision (Borges et al. 2015) characterized by four cone photoreceptor opsin pigments with distinct spectral sensitivities, but it appears that owls have a reduced capacity to discriminate colors. Our genomic data for the color vision system in owls are largely consistent with the results of a retinal microspectrophotometry study (Bowmaker and Martin 1978), retinal transcriptome analyses (Wu et al. 2016), and a recent genomic study of avian visual opsins (Borges et al. 2015). Specifically, the absence of *SWS1*, which absorbs light in the violet/ultraviolet (Davies et al. 2012), in both *S. o. caurina* and *T. alba* is corroborated by the absence of a violet/ultraviolet-sensitive photopigment in *S. aluco* (Bowmaker and Martin 1978), the lack of *SWS1* retinal mRNA transcripts in a tytonid and species from all three of the strigid subfamilies (Wu et al. 2016), and a genomic analysis of *T. alba* that also failed to find *SWS1* in the genome assembly (Borges et al. 2015). In our *S. o. caurina* assembly we were able to locate, albeit on separate scaffolds, the genes that flank *SWS1* in other avian taxa, but not *SWS1* itself. More data is needed to confirm whether there are *SWS1* remnants in the *S. o. caurina* and *T. alba* genomes and their absence in the current assemblies is simply due to assembly incompleteness or errors. However, together the data accumulated to date strongly indicate that owls lack *SWS1*, potentially since their most recent common ancestor, leading to a reduced capacity for color discrimination. The loss of *SWS1* is highly unusual in Aves (Borges et al. 2015). Other than in owls, it has only been inferred to have been lost in the nocturnal North Island brown kiwi (*Apteryx mantelli*) (Le Duc et al. 2015). In contrast, it has occurred repeatedly in nocturnal, subterranean, and marine mammals (Jacobs 2013; Emerling et al. 2015) as well as in the crocodilians, a lineage believed to have undergone an extensive period of nocturnal adaptation (Walls 1942; Emerling 2017a).

The inactivation of *Rh2* in *T. alba* was previously suggested (Borges et al. 2015) and we confirmed this result with the two premature stop codons and two frameshift indels we found in the gene sequence. Additionally, there is evidence that the retinal transcriptome of a congener, *T. longimembris*, does not include *Rh2* transcripts (Wu et al. 2016). The intact copy of *Rh2* in our *S. o. caurina* genome, the transcription of this gene in multiple strigid species (Wu et al. 2016), and the expression of a cone pigment consistent with the Rh2 protein in *S. aluco* (Bowmaker and Martin 1978) all support the hypothesis that *Rh2* was lost uniquely in the tytonid lineage and not across Strigiformes (Wu et al. 2016). Among avian species, *Rh2* is also inactivated in the kiwi *A. mantelli* (Le Duc et al. 2015) as well as in the Adélie (*Pygoscelis adeliae*) and emperor penguins (*Aptenodytes forsteri*) (Li et al. 2014; Borges et al. 2015), two marine predators that frequently feed at great depths under dim-light conditions. A third penguin species, the Humboldt penguin (*Spheniscus humboldti*) lacks cones with a peak absorbance typical of *Rh2* (Bowmaker and Martin 1985). The loss of *Rh2* occurred in several other vertebrate groups that are thought to have experienced long periods of inhabiting dim-light environments, including stem Mammalia (Walls 1942; Davies et al. 2007; Gerkema et al. 2013), Crocodilia (Emerling 2017a), and snakes (Reptilia: Serpentes) (Castoe et al. 2013; Vonk et al. 2013; Simões et al. 2015; Emerling 2017b).

The apparent absence of *SWS2* and *LWS* in *T. alba* is likely due to the assembly being incomplete. These genes are in tandem in *A. carolinensis* and *X. laevis*, but the avian assemblies in Ensembl version 86 (Yates et al. 2016) contain *SWS2* and *LWS* on separate small contigs and not adjacent to other genes. This is consistent with our recovery of only partial *SWS2* and *LWS* in *S. o. caurina* and previous difficulties in assembling full *SWS2* and *LWS* sequences in dozens of other avian genomes (Borges et al. 2015; Le Duc et al. 2015), which may be attributable to the high GC content of these genes (Borges et al. 2015). Researchers recovered intact *SWS2* and *LWS* mRNAs in the retinal transcriptomes of five strigid and one tytonid species (Wu et al. 2016) and have demonstrated that the tawny owl (*S. aluco*) expresses photoreceptor

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pigments with peak absorptions consistent with *SWS2* and *LWS* (Bowmaker and Martin 1978), suggesting that *SWS2* and *LWS* are likely retained in owls.

Together, the confluence of data from genomics, transcriptomics, and retinal microspectrophotometry suggests that *SWS1* was likely lost in stem Strigiformes, which resulted in a reduction in the degree of color vision from tetrachromacy to trichomacy by the time of the last common ancestor of owls. *Rh2* became subsequently inactivated in Tytonidae, resulting in further reduced capacity for color discrimination (dichromacy) in this family. Owls, kiwis, and penguins represent the few known avian taxa that deviated from the ancestral avian state of tetrachromatic color vision, likely as a result of an increased dependence on highly sensitive rod photoreceptors for foraging in low-light conditions.

The inactivation (*T. alba*) or deletion (*S. o. caurina*) of the gene encoding pinopsin (*OpnP*) may have resulted in the loss of direct photosensitivity of the pineal gland in owls. Pinopsin is expressed in the pineal gland of birds (Okano et al. 1994) and likely regulates the daily rhythms of melatonin synthesis. Owls have a relatively small and simple pineal with little response to differences in luminance (Taniguchi et al. 1993), which suggests that, similar to mammals, the gland may receive photic input indirectly from the eyes (Falcón et al. 2009). *OpnP* is also inactivated in the penguins *P. adeliae* and *A. forsteri* (Li et al. 2014), but it otherwise appears intact across Aves (Borges et al. 2015). Notably, the loss of pinopsin has also occurred in the historically dim-light-environment-inhabiting Mammalia, Crocodilia, and Serpentes (Walls 1942; Gerkema et al. 2013; Emerling 2017a, 2017b). Crocodilians appear to lack a pineal gland entirely (Roth et al. 1980), whereas mammals have a pineal gland that has moved from a more superficial to a deeper position in the brain (Falcón et al. 2009), presumably resulting in a loss of photosensitivity. Together these data suggest that the loss of direct photosensitivity of the pineal gland is a common theme in amniotes (Tetrapoda: Amniota) that experience minimal exposure to light.

Although we found several putative inactivating mutations in *Opn4m*, these are unlikely to have led to complete loss of function. The shared 4 nt mutation in *T. alba* and *S. o. caurina* suggests that it was inherited from the common ancestor of Strigiformes. If this mutation disrupted the function of *Opn4m* in the common ancestor of Strigiformes, then this gene sequence should have been evolving neutrally in all of the descendant lineages. However, Strigidae and Tytonidae split ~45 million years ago (Prum et al. 2015) yet each ortholog has only accumulated a single additional putative inactivating mutation, both of which are downstream of exon 8. Our dN/dS ratio analyses of crown owl branches yielded an $\omega < 1$ ($\omega = 0.45$), which is consistent with the hypothesis that *Opn4m* remains functional in owls. Furthermore, we were able to assemble *Opn4m* from the retinal mRNA sequences from six additional owls (five strigid and one tytonid), which indicates that *Opn4m* is still being transcribed in the eyes of those species. We found evidence of multiple *Opn4m* isoforms in the avian retinal transcriptome sequences and the genomic sequences of several other avian taxa possessed putative inactivating mutations. These potentially inactivating mutations were almost all distributed on or after exon 8. Notably, when we used the lowest sensitivity setting of the Geneious aligner to map *Opn4m* BLAST hits from the avian retinal transcriptomes, we primarily obtained assembled sequences that terminated after exon 8. Previous work has found multiple *Opn4m* isoforms in vertebrates (Verra et al. 2011; Hughes et al. 2012). Our results suggest loss of some of these isoforms in owls and other birds. *Opn4m* is involved in entraining circadian rhythms in mammals via the pineal gland, in part, as well as in regulating pupil diameter (Hankins et al. 2008). Given the diminished importance of the pineal gland in owls, alteration of the circadian function of *Opn4m* is a possibility.

CYP2J19 has recently been implicated as the carotenoid ketolase responsible for synthesizing red carotenoids in birds (Lopes et al. 2016; Mundy et al. 2016; Emerling 2017c). Carotenoids, in addition to being involved in pigmentation of avian skin and feathers, are located in oil droplets anterior to the photosensitive outer segments of cone photoreceptors. These oil droplets fine-tune color vision by absorbing shorter wavelengths and reducing spectral overlap between cone visual pigments (Vorobyev 2003). However, these droplets also reduce the number of photons that reach cone photoreceptors and, therefore, may be less beneficial under dim-light conditions. Among owls, *S. aluco*, *Athene noctua* (little owl), and *Asio flammeus* (short-eared owl) are known to possess red cone oil droplets, whereas *Strix uralensis* (Ural owl), *Bubo scandiacus* (snowy owl), and *T. alba* lack them (Erhard 1924; Yew et al. 1977; Bowmaker and Martin 1978; Gondo and Ando 1995). In *S. aluco*, the red oil droplets are limited to <1% of the cone photoreceptor population (Bowmaker and Martin 1978), which is an extremely low proportion compared with other avian species (Bowmaker 1980; Partridge 1989). Additionally, there is recent evidence that *CYP2J19* is inactivated in *T. alba*, is transcribed as a pseudogene in the retinal transcriptome of *Asio otus* (long-eared owl), and is transcribed at low levels in five other owl species as compared with the level observed in diurnal outgroup avian taxa (Emerling 2017c). Among non-owl Aves, the absence of red cone oil droplets has only been reported in two penguin species, *S. humboldti* (Bowmaker and Martin 1985) and *Aptenodytes patagonicus* (Gondo and Ando 1995). Among nonowls, *CYP2J19* is inactivated in the penguins *P. adeliae* and *A. forsteri* as wells as in the kiwi *A. mantelli* (Emerling 2017c), which all forage under dim-light conditions. The *CYP2J19* pseudogene reported here for *S. occidentalis caurina* provides further evidence that owls have repeatedly been losing red carotenoid oil droplets in parallel, potentially to maximize retinal sensitivity in their predominantly nocturnal niche.

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Perhaps what is most notable about the loss of light-associated genes in Strigiformes is not the fact that it has occurred, but that it has not ensued to the same extent as in other historically dim-light-adapted vertebrates. Of the nineteen genes we examined, all but one (*CYP2J19*) were likely present in the common ancestor of amniotes (Gerkema et al. 2013; Osborn et al. 2015; Twyman et al. 2016). Excluding *CYP2J19*, mammals lost nine (Mammalia: Marsupialia and Monotremata) to ten of these genes (Mammalia: Placentalia) during a hypothesized nocturnal or mesopic bottleneck (Walls 1942; Heesy and Hall 2010; Davies et al. 2012; Gerkema et al. 2013) and crocodilians lost seven during a similarly hypothesized period of dim-light adaptation (Walls 1942; Emerling 2017a). Among squamates (Reptilia: Squamata), snakes lost seven of these genes during a putative nocturnal and/or fossorial period early in their history, whereas the largely nocturnal geckos lost six (Walls 1942; Emerling 2017b). As for owls, tytonids have lost three of the light-associated genes we examined (*SWS1*, *Rh2*, *OpnP*), whereas strigids have lost only two (*SWS1*, *OpnP*).

Conclusions

We report the first genome of a member of Strigidae, the largest family of owls. We anticipate that this draft whole genome assembly will be useful to those studying the genetics, demography, and conservation of the spotted owl and related taxa. It will be of particular use in genetic identification of hybrid spotted/barred owls (*S. occcidentalis* × *varia*) and in ascertaining the frequency of hybridization between these two species in the forests of western North America. The phylogenetic position of owls within Neoaves is at the base of a large clade containing mousebirds (Coliiformes), cuckoo-rollers (Leptosomiformes), trogons (Trongoniformes), hornbills (Bucerotiformes), woodpeckers (Piciformes), and kingfishers (Coraciiformes) (Jarvis et al. 2014; Prum et al. 2015). This placement of owls suggests that our spotted owl genome assembly will be useful in genomic studies that span a substantial component of avian morphologic diversity and life history strategies.

Despite potentially more than 45 million years of dim-light specialization in Strigiformes, owls have retained a diverse array of nonvisual opsin pigments and mechanisms to protect against ultraviolet photo-oxidative damage. Although tytonids have a reduced color vision capacity that is similar to ancestral mammals, crocodilians, and snakes, strigids have retained trichromatic color vision akin to that of humans. Many light-associated gene functions have been maintained in owls, perhaps enabling activities during daylight, a time when most owls are presumed to be generally inactive. It appears that what many consider the quintessential nocturnal birds are not as independent of light as are other nocturnal or crepuscular amniote lineages.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

Acknowledgments

We thank WildCare, San Rafael for graciously providing us with blood samples from Sequoia. We thank the Cincinnati Museum Center for providing a barred owl (*S. varia*) tissue sample. We extend special thanks to J. Graham Ruby for his assistance with initial project design and assembly methodology. We thank Anna Sellas for assistance with lab work as well as Ke Bi and Stefan Prost for helpful methodological discussions. We generated genetic data at the Center for Comparative Genomics, California Academy of Sciences. Genewiz, Inc. (USA) constructed and sequenced the Nextera (Illumina, USA) large-insert mate-pair libraries. Research performed under University of California, Berkeley Institutional Animal Care and Use Committee approval number R317. This work was supported by funding from Michael and Katalina Simon [to J.P.D.]; the Louise Kellogg Fund, Museum of Vertebrate Zoology, University of California, Berkeley [to Z.R.H.]; the National Science Foundation Graduate Research Fellowship [DGE 1106400 to Z.R.H.]; the Howard Hughes Medical Institute [to J.L.D.]; and the National Science Foundation Postdoctoral Research Fellowship [DBI 1523943 to C.A.E.]. Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. This work used data produced by the Vincent J. Coates Genomics Sequencing Laboratory at the University of California, Berkeley, supported by National Institutes of Health S10 Instrumentation Grants [S10 RR029668, S10 RR027303]. Publication made possible in part by support from the Berkeley Research Impact Initiative (BRII) sponsored by the UC Berkeley Library.

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Supplementary Article for:

Northern spotted owl (*Strix occidentalis caurina*) genome: divergence with the barred owl (*Strix varia*) and characterization of light-associated genes

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1 Supplementary Material and Methods

1.1 Nextera350nt library

1.1.1 We intended this library to be a Nextera-sheared library with a small insert size. We isolated DNA using a Gentra Puregene Kit (Qiagen) following the protocol entitled "Protocol: DNA Purification from Tissue Using the Gentra Puregene Tissue Kit" (Qiagen). We used 50 ng of the DNA to prepare a genomic library using a Nextera DNA Sample Prep Kit (Illumina-compatible) (Epicentre). After tagmentation, we cleaned the reaction with a DNA Clean & Concentrator -5 kit (Zymo Research). We amplified the reaction for 5 cycles of PCR using a Nextera DNA Sample Prep Kit (Illumina-compatible) (Epicentre) and the Nextera PCR Enzyme (Epicentre). We then cleaned the reaction with a DNA Clean & Concentrator -5 kit (Zymo Research). We used a LabChip XT DNA 750 Assay Kit on a LabChip XT (PerkinElmer) automated nucleic acid fractionation system to select library fragments in the size range of 375-600 nt, which, after subtracting the 141 nt of adapters, corresponds to an average fragment size of 346.5 nt. We performed a final PCR using 5 μL Klentaq LA 10X Buffer with MgCl (Sigma-Aldrich), 1 μL 12.5 μM dNTPs, 1 μL each of two Illumina-adapter-compatible primers at 10 μM, 1 μL KlenTaq LA DNA Polymerase Mix (Sigma-Aldrich), 5 μL library off of LabChip, and water to make a 50 μL reaction volume. We ran the PCR at 94°C for 2 min; then 5 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 3 min; and we performed a final extension at 72°C for 5 min. We removed the PCR products after the final extension and and then cleaned them using a DNA Clean & Concentrator -5 kit (Zymo Research). We obtained one lane of 100 nt paired-end data using a TruSeq PE Cluster Kit v2-cBot-HS kit and a TruSeq SBS v2-HS

kit on a HiSeq 2000 (Illumina) and a second lane of 100 nt paired-end data using a TruSeq PE Cluster Kit v3-cBot-HS kit and a TruSeq SBS v3-HS kit on a HiSeq 2000 (Illumina).

1.2 Nextera700nt library

1.2.1 We attempted to construct a Nextera-sheared library with a moderate insert size. We isolated DNA using a Gentra Puregene Kit (Qiagen) and used 50 ng to prepare a genomic library using a Nextera DNA Sample Preparation Kit (Illumina). After tagmentation, we cleaned the reaction with a DNA Clean & Concentrator -5 kit (Zymo Research). We amplified the reaction for 5 cycles of PCR using a KAPA Library Amplification kit (KAPA Biosystems) and then cleaned the reaction with a DNA Clean & Concentrator -5 kit (Zymo Research). We used a BluePippin (Sage Science) to select library fragments in the size range of 734-934 nt, which, after subtracting the 134 nt of adapters, corresponded to selecting an average insert size of 700 nt. We performed a real-time PCR (rtPCR) using a KAPA Real-Time Library Amplification Kit (KAPA Biosystems) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) to amplify the library. We amplified the library with 6 cycles PCR and then cleaned the PCR products with a DNA Clean & Concentrator -5 kit (Zymo Research). We lastly assessed the library fragment size distribution with a 2100 BioAnalyzer (Agilent Technologies) and the concentration of double-stranded DNA material with a Qubit 2.0 Flurometer (Invitrogen). We obtained one lane of 150 nt paired-end data sequenced on a HiSeq 2500 (Illumina) in rapid mode.

1.3 Nextera550nt library

1.3.1 We aimed to construct a Nextera-sheared library with overlapping reads, which could be merged into long fragments. We isolated DNA using a Gentra Puregene Kit (Qiagen) and

used 50 ng to prepare a genomic library using a Nextera DNA Sample Preparation Kit (Illumina). After tagmentation, we cleaned the reaction with a DNA Clean & Concentrator -5 kit (Zymo Research). We amplified the reaction for 5 cycles of PCR using a KAPA Library Amplification kit (KAPA Biosystems) and then cleaned the reaction with a DNA Clean & Concentrator -5 kit (Zymo Research). We then used a BluePippin (Sage Science) to select library fragments in the size range of 634-709 nt, which, after subtracting the 134 nt of adapters, corresponded to selecting an average insert size of 537.5 nt. We assessed the library fragment size distribution with a 2100 BioAnalyzer (Agilent Technologies). We cleaned the size-selected product with 0.6X Agencourt AMPure XP (Beckman Coulter) magnetic beads to remove adapter dimer of approximately 250 nt in size. We then performed a real-time PCR (rtPCR) using a KAPA Real-Time Library Amplification Kit (KAPA Biosystems) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) to amplify the library. We amplified the library with 8 cycles PCR and then cleaned the PCR products with a DNA Clean & Concentrator -5 kit (Zymo Research). We lastly assessed the library fragment size distribution with a 2100 BioAnalyzer (Agilent Technologies) and the concentration of double-stranded DNA material with a Qubit 2.0 Flurometer (Invitrogen). We obtained one lane of 300 nt paired-end data sequenced using a MiSeq Reagent Kit v3 on a MiSeq (Illumina). We obtained a second lane of 375 nt read 1 and 225 nt read 2 for a total of 600 nt of paired-end read data sequenced using a MiSeq Reagent Kit v3 on a MiSeq (Illumina).

1.4 noPCR550nt library

1.4.1 We extracted genomic DNA from blood using a DNeasy Blood & Tissue Kit (Qiagen). We sheared 4,460 ng genomic DNA in 130 µL in a microTUBE AFA Fiber Pre-Slit

Snap-Cap tube (Covaris) using a M220 focused-ultrasonicator (Covaris) targeting 550 nt as the center of the fragment distribution. We used peak incident power 50 W, 20% duty factor, 200 cycles per burst, and 45 s treatment time at 20°C. We then removed small fragments and concentrated the sheared material using a DNA Clean & Concentrator -5 kit (Zymo Research). We next constructed a genomic library by using a TruSeq DNA PCR-Free Library kit (Illumina) and following the manufacturer's protocol, including the use of bead-based size selection to remove large and small DNA fragments in succession to target a mean fragment size of 550 nt. We assessed the concentration of double-stranded DNA material in the final library with a Qubit 2.0 Flurometer (Invitrogen).

1.5 900ntPCR library

1.5.1 We extracted genomic DNA from blood using a DNeasy Blood & Tissue Kit (Qiagen). We sheared 4,580 ng genomic DNA in 130 μL in a microTUBE AFA Fiber Pre-Slit Snap-Cap tube (Covaris) using a M220 focused-ultrasonicator (Covaris) targeting 900 nt as the center of the fragment distribution. We used peak incident power 50 W, 5% duty factor, 200 cycles per burst, and 70 s treatment time at 20°C. We then removed small fragments and concentrated the sheared material using a DNA Clean & Concentrator -5 kit (Zymo Research). We next constructed a genomic library by using a TruSeq DNA PCR-Free Library kit (Illumina) and following the manufacturer's protocol, except that we only performed a bead-based size selection to remove small fragments and not large fragments. We used a 0.45X bead to sample ratio in order to eliminate fragments smaller than approximately 700 nt. Following A-tailing and prior to adapter ligation, we took 10% of the sample (by volume) and separated it from the noPCR aliquot for use in a PCR-amplified library. We ligated adapters to these two aliquots separately and cleaned

the finished ligations with a DNA Clean & Concentrator -5 kit (Zymo Research). We then only went forward with the aliquot for use in a PCR-amplified library. We used a BluePippin (Sage Science) to select library fragments in the size range of 800-1100 nt, which, after subtracting the 121 nt of adapters, corresponded to selecting an average insert size of 829 nt. We next cleaned the eluted material with a DNA Clean & Concentrator -5 kit (Zymo Research) and then performed real-time PCR (rtPCR) using a KAPA Real-Time Library Amplification Kit (KAPA Biosystems) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) to amplify the library. We amplified the library with 11 cycles PCR and then cleaned the PCR products with 1X Agencourt AMPure XP (Beckman Coulter) magnetic beads. We lastly assessed the library fragment size distribution with a 2100 BioAnalyzer (Agilent Technologies) and the concentration of double-stranded DNA material with a Qubit 2.0 Flurometer (Invitrogen).

1.6 Hydroshear library

1.6.1 We isolated DNA using a Gentra Puregene Kit (Qiagen) and used a Hydroshear DNA Shearing Device (GeneMachines) to shear 25 µg in DNA in 100 µL volume with 30 cycles of shearing using speed code 3. We checked the sheared DNA on a 1% agarose gel and saw that fragments had been sheared between 400-1000 nt. We additionally mechanically sheared the DNA by performing 15 passes through a 28 gauge x 1/2 inch needle attached to a 1 cc U-100 Insulin Syringe (Becton, Dickinson and Company). We performed end-repair using 4266 ng sheared DNA in an End-It DNA End-Repair Kit (Epicentre). We incubated the reaction at room temperature for 45 minutes and then inactivated the enzyme by heating to 72°C for 10 minutes followed by cleaning with a DNA Clean & Concentrator -5 kit (Zymo Research). We then added 3’ A tails in a

reaction with 2 μL 10X NEBuffer 2, 0.5 μL 100 mM dATP (Invitrogen), 1 μL Klenow Fragment (3'→5' exo-) (NEB), and 16.5 μL cleaned end-repaired product. We incubated for 45 min at 37°C and then 20 min at 75°C to inactivate the enzyme. We cleaned the reaction with a DNA Clean & Concentrator -5 kit (Zymo Research). We then ligated Illumina-compatible adapters using 1 μL 10X Fast-Link Ligation Buffer (Epicentre), 1 μL 10 mM ATP (Epicentre), 5 μL of end-repaired DNA (0.7835 μg), 2 μL of annealed Illumina-compatible adapters at 10 μM (Integrated DNA Technologies), and 1 μL Fast-Link DNA Ligase (Epicentre) for 10 μL total reaction volume. We incubated the ligation reaction overnight at 16°C and then used 1.5X Agencourt AMPure XP (Beckman Coulter) magnetic beads to clean the ligase reaction and remove any extra adapters. We performed a PCR using 10 μL Klentaq LA 10X Buffer with MgCl (Sigma-Aldrich), 2 μL 12.5 μM dNTPs, 2 μL each of two Illumina-adapter-compatible primers at 10 μM, 2 μL KlenTaq LA DNA Polymerase Mix (Sigma-Aldrich), half of the cleaned ligase reaction in 10 μL, and water to make a 100 μL reaction volume. We ran the PCR in two 50 μL aliquots at 94°C for 5 min; then 2 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 3 min; and we performed a final extension at 68°C for 5 min. We removed the PCR products after the final extension and and then cleaned them using a DNA Clean & Concentrator -5 kit (Zymo Research). We used a LabChip XT DNA 750 Assay Kit on a LabChip XT (PerkinElmer) automated nucleic acid fractionation system to select library fragments in the size range of 600-700 nt. We performed a final PCR using 5 μL Klentaq LA 10X Buffer with MgCl (Sigma-Aldrich), 1 μL 12.5 μM dNTPs, 1 μL each of two Illumina-adapter-compatible primers at 10 μM, 1 μL KlenTaq LA DNA Polymerase Mix (Sigma-Aldrich), 5 μL library off of LabChip,

and water to make a 50 µL reaction volume. We ran the PCR at 94°C for 2 min; then 17 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 3 min; and we performed a final extension at 72°C for 5 min. We removed the PCR products after the final extension and and then cleaned them using a DNA Clean & Concentrator -5 kit (Zymo Research). We next assessed the library fragment size distribution with a 2100 BioAnalyzer (Agilent Technologies) and the concentration of double-stranded DNA material with a Qubit 2.0 Flurometer (Invitrogen).

1.7 noPCR550nt, 900ntPCR, and Hydroshear libraries

1.7.1 We pooled the barcoded noPCR550nt, 900ntPCR, and Hydroshear libraries equimolarly and we obtained 350 nt read 1 and 250 nt read 2 for a total of 600 nt of paired-end read data from one lane (approximately ⅓ of one lane per library) using a 600-cycle MiSeq Reagent Kit v3 on a MiSeq (Illumina).

1.8 MP4kb, MP7kb, and MP11kb libraries

1.8.1 We constructed and sequenced three large-insert mate-pair libraries. We isolated DNA using a Gentra Puregene Kit (Qiagen) and sent 41.3 µg to GENEWIZ (www.genewiz.com). We requested barcoded mate-pair libraries with insert sizes of 4 kb, 6 kb, and 11 kb constructed using the Nextera Mate Pair Sample Preparation Kit (Illumina). GENEWIZ followed the procedure detailed in the Nextera Mate Pair Sample Preparation Guide (Illumina, Part # 15035209 Rev. C, January 2013). Traces obtained using a 2100 BioAnalyzer (Agilent Technologies) showed the centers of the distributions of the sheared fragmentsthat went into the circularization step of the three mate-pair libraries as 4.2 kb, 7.1 kb, and 10.7 kb. GENEWIZ pooled the three libraries equimolarly

and we obtained one lane (approximately ⅓ of one lane per library) of 100 nt paired-end data sequenced on a HiSeq 2000 (Illumina).

1.9 Trimming - long-insert mate-pair data

1.9.1 We trimmed the Nextera mate-pair data using NxTrim version 0.2.3-alpha (O'Connell 2014; O'Connell et al. 2015), which required BOOST version 1.57.0 (http://www.boost.org). When running NxTrim, we used the "--preserve-mp" flag to prefer mate pair reads as output even if paired-end reads would be longer. NxTrim utilizes the position of the junction identifier sequence in Nextera mate-pair data to classify reads of mate pair libraries as true mate pair reads, paired-end reads, or singleton reads.

1.9.2 We trimmed adapters and low quality bases separately for the resulting mate-pair data, paired-end reads, and singleton reads using Trimmomatic version 0.32 (Bolger et al. 2014). We trimmed adapters using options "ILLUMINACLIP:<fasta of Illumina adapter sequences >:2:30:10". We removed low quality bases from the beginning and end of the reads using the following options: LEADING:3 TRAILING:3 to remove bases below Phred 3. We trimmed off low quality sequence portions using: SLIDINGWINDOW:4:17, which trimmed the read when the average quality over 4 basepairs dropped below Phred 17. Finally, we trimmed reads less than 36 basepairs in length using "MINLEN:36".

1.10 Trimming - short-insert paired-end data

1.10.1 We first trimmed adapters from all non-mate-pair libraries using Trimmomatic version 0.32 (Bolger et al. 2014). We used the ILLUMINACLIP function with the following options: <fasta of Illumina adapter sequences >:2:30:10.

1.10.2 Since substantial portions of the paired-end reads from all of the libraries, except the Nextera700nt library were overlapping, we joined overlapping paired reads using the BBMerge tool in the BBMap tool suite version 34.00 (Bushnell 2014). We merged overlapping reads using the options "minoverlapinsert=110 mininsert=110 strict=t" for the datasets Nextera350nt lane 1 and Nextera350nt lane 2, We used the options "minoverlapinsert=400 mininsert=400 strict=t" for the datasets Nextera550nt lane 1, Nextera550nt lane 2, noPCR550nt, and PCR900nt, which had longer read lengths.

1.10.3 We then performed quality trimming using Trimmomatic version 0.32 (Bolger et al. 2014). We removed low quality bases from the beginning and end of the reads using the options “LEADING:3 TRAILING:3” to remove bases below Phred 3. We trimmed off low quality sequence portions using “SLIDINGWINDOW:4:17”, which trimmed the read when the average quality over 4 basepairs dropped below Phred 17. Finally, we trimmed reads less than 36 basepairs in length using “MINLEN:36”.

1.11 Error-correction

1.11.1 Since we trimmed using a moderately low quality threshold, we used the k-mer-based error corrector in the SOAPdenovo2 toolkit, SOAPec version 2.01 (Luo et al. 2012), to correct sequence errors. We first used the KmerFreq_HA tool to create a k-mer frequency spectrum with default options except “-k 27 -L 600”, which indicate that we used a k-mer size of 27 for creating the frequency spectrum and the maximum read length was 600 nt. We then used the Corrector_HA tool along with the k-mer frequency spectrum that we created to correct all of our trimmed reads using default options except “-k 27 -r 36”, which indicate that we used a k-mer size of 27 for the error correction and kept trimmed reads as short as 36 nt.

1.12 Single-end data

1.12.1 In each stage of the trimming, merging, and error-correction process, some reads previously paired became unpaired due to the loss of their paired read in a trimming step. We handled the single-end reads separate from the paired reads and subjected them to the same adapter, quality trimming, and error-correcting steps as the reads that remained paired. We used all of these single read sets in the final assembly.

1.13 Read processing variation for some preliminary assemblies

1.13.1 For a trim level of an average Phred 7 or 28, the only difference from the methodology described above was that we trimmed off low quality sequence portions using Trimmomatic with the parameter "SLIDINGWINDOW:4:7" or "SLIDINGWINDOW:4:28", respectively.

1.13.2 We did not apply the error-correction process to reads trimmed to an average Phred 28.

1.13.3 For some preliminary assemblies, we did not merge overlapping paired-end reads. This entailed leaving out the BBMerge step described above, but still performing adapter and quality trimming as noted.

1.14 Genome size

1.14.1 Genome size data estimated from flow cytometry measurement of red blood cells exist for two *S. occidentalis* congeners of, *S. aluco* and *S. nebulosa*. *Strix aluco* has a C-value of 1.59 pg (De Vita et al. 1994), which is approximately 1.56 Gnt (Doležel et al. 2003). *Strix nebulosa* has a C-value of 1.65 pg (Vinogradov 2005), which is approximately 1.61 Gnt (Doležel et al. 2003).

1.14.2 We ran Preqc (Simpson 2014), a module within SGA version 0.10.14 (Simpson & Durbin 2010, 2016), which used Google SparseHash library version 2.0.2 (google-

sparsehash@googlegroups.com 2012), zlib version 1.2.8 (Gailly & Adler 2013) and BamTools version 2.4.0 (Barnett et al. 2011, 2015) requiring CMake version 3.2.3 (Hoffman & Martin 2003; Kitware 2015), and on the 150 nt paired-end reads from the Nextera700nt dataset to estimate the genome size. Preqc estimated the genome size by sampling 20,000 reads and counting the frequency of *k*-mers of length 31 nt while applying a correction for sequencing errors.

1.15 Assembly

1.15.1 We used SOAPdenovo2 version 2.04 (Luo et al. 2012) to assemble the genome. We performed numerous trial runs experimenting with different k-mer values and parameters. We utilized the insert size estimated in the output of initial, trial assemblies to refine our estimation of the insert sizes for our libraries and used these refined values as input into subsequent assembly configuration files (Table S1). We settled on using the default parameters other than the options "SOAPdenovo-127mer all -N 1500000000 -K 23 -m 127 -k 65 -d 1 -R -F". These options indicate that we used the 127 k-mer version of the assembler and ran the assembly using multiple k-mer sizes starting at 23 and ending with a maximum of 127, we gave an estimated genome size of 1.5 Gnt, we allowed reads as small as 65 nt to map to contigs during scaffolding, we ignored singleton k-mers, we tried to resolve repeats with reads, and we attempted to fill gaps in scaffolds.

1.15.2 In our configuration files for all of the preliminary assemblies, we used the default minimum alignment lengths between a read and contig (32 for paired-end reads, 35 for mate-pair reads) and the default minimum pair number cutoffs (3 for paired-end reads, 5 for mate-pair reads).

1.15.3 We used dupchk (Henderson & Hanna 2016a), which utilized the first and last 21 nt of each read as a read fingerprint, to check for sequence duplication in each sequenced library.

1.16 Preliminary assembly assessment

1.16.1 In order to compare our preliminary assemblies, we removed contigs / scaffolds <= 300 nt in order to remove any unassembled reads from the assembly. We calculated the contig and scaffold N50 as well as the number of scaffolds in various length classes using scafN50 (Henderson & Hanna 2016d). We calculated the total length of the assembly, the % Ns, and the total number of scaffolds using scafSeqContigInfo (Henderson & Hanna 2016e). We were conservative and separated scaffolds into contigs at each N in the sequence, which is the default option for scafSeqContigInfo (Henderson & Hanna 2016e).

1.16.2 We then used CEGMA version 2.5 (Parra et al. 2007), which required GeneWise from the Wise2 version 2.2.3-rc7 package (Birney; Birney et al. 2004), HMMER version 3.0 (http://hmmer.org), geneid version 1.4.4 (Guigó 1998; Blanco et al. 2011), and NCBI's BLAST+ version 2.2.25 (Altschul et al. 1997; Camacho et al. 2009), to annotate a set of highly conserved eukaryotic genes in our assembly and thereby obtain an assessment of the quality and completeness of each assembly. In order to install CEGMA's GeneWise dependency, we followed the source code modification recommendations documented by Markus Grohme (http://korflab.ucdavis.edu/datasets/cegma/ubuntu_instructions_1.txt) and the Homebrew Science GeneWise formula (https://github.com/Homebrew/homebrew-science/blob/master/genewise.rb).

1.17 Determination of final assembly

1.17.1 We examined multiple statistics in choosing our final assembly. We valued high contig and scaffold N50 values, low % Ns in the sequence, a low total number of scaffolds, larger numbers of long scaffolds, and completeness as reflected in the number of conserved genes found by the CEGMA pipeline. We decided that the assembly that had the best statistics across these categories was assembly 4 (Table 2) and we went forward with this assembly as our final assembly.

1.18 Gap closing

1.18.1 We found that using the "-F" flag to fill gaps using the SOAPdenovo2 version 2.04 (Luo et al. 2012) *de novo* assembler was ineffective at gap filling during the assembly. We then filled gaps using the gap closing tool in the SOAPdenovo2 toolkit, GapCloser version 1.12-r6 (Luo et al. 2012), with the default options other than "-l 600" to specify that our longest read length was 600 nt. The program output a warning stating that the maximum supported read length was 155 nt and that it would use that setting for the analysis. We assumed that the program just used the first 155 nt of reads with a total length exceeding 155 nt.

1.18.2 The gap-closed assembly contained many contigs and/or scaffolds under 1000 nt in length, a substantial portion of which appeared to be unassembled reads. We used ScaffSplitN50s (Henderson & Hanna 2016c) to compare the continuity statistics resulting after removing contigs / scaffolds of lengths 300, 500, and 1,000 nt as well as when using N blocks of lengths 1, 5, 10, 15, 20, and 25 to separate contigs within scaffolds. Based on these results, we removed all contigs and scaffolds less than 1000 nt for downstream analyses.

1.19 Final assembly stats

1.19.1 We used CEGMA version 2.5 (Parra et al. 2007), which required GeneWise from the Wise2 version 2.2.3-rc7 package (Birney; Birney et al. 2004), HMMER version 3.0 (http://hmmer.org), geneid version 1.4.4 (Guigó 1998; Blanco et al. 2011), and NCBI's BLAST+ version 2.3.0 (Altschul et al. 1997; Camacho et al. 2009), to annotate a set of highly conserved eukaryotic genes in our assembly and thereby obtain an assessment of the quality and completeness of the assembly. We ran CEGMA with default parameters other than specifying "--vrt" to optimize the searches for a vertebrate genome.

1.19.2 We used BUSCO version 1.1b1 (Simão et al. 2015a; Simão et al. 2015b), which used NCBI's BLAST+ version 2.2.28 (Altschul et al. 1997; Camacho et al. 2009), HMMER version 3.1b2 (http://hmmer.org), and AUGUSTUS version 3.2.1 (Keller et al. 2011; Stanke 2015) to assess the assembly quality by searching for conserved orthologs. We ran BUSCO with default genome mode parameters other than specifying "vertebrata" as the evolutionary lineage with the option "-l" and using "-sp chicken" to employ the AUGUSTUS parameters optimized for the chicken genome.

1.20 Contamination assessment

1.20.1 We performed a local alignment of all scaffolds in NSO-wgs-v0 to a copy the NCBI nucleotide database (nt) that we downloaded on 24 June 2016 (Clark et al. 2016; NCBI Resource Coordinators 2016) using NCBI's BLAST+ version 2.3.0 tool BLASTN (Altschul et al. 1997; Camacho et al. 2009) with default parameters other than "-outfmt 10 -num_alignments 5 -max_hsps 1". We used these parameters to limit to 5 the maximum number of alignments to unique subjects output and to limit to 1 the number of outputted alignments per subject. This allowed us to examine the top 5 alignments to

different subject sequences and ascertain whether those subject sequences were obtained from vertebrate or non-vertebrate organisms.

1.20.2 In order to parse the taxonomy of the subject sequences in the alignment output, we obtained the a local copy of the NCBI taxonomy database using NCBI's BLAST+ version 2.3.0 script, update_blastdb.pl with the parameters "--passive --timeout 300 --force --verbose taxdb". We also downloaded the files taxdump.tar.gz and gi_taxid_nucl.dmp.gz from NCBI (ftp://ftp.ncbi.nlm.nih.gov/pub/taxonomy) (Clark et al. 2016; NCBI Resource Coordinators 2016). We then used GItaxidIsVert (Henderson & Hanna 2016b) with default options other than using the parameter "-n" to filter the alignment output for non-vertebrate alignments.

1.20.3 We used the web version of NCBI's BLAST+ version 2.4.0 tool BLASTN (Altschul et al. 1997; Camacho et al. 2009) with default parameters.

1.21 Mitochondrial genome identification

1.21.1 We searched NSO-wgs-v1 (not repeat-masked, all contigs / scaffolds < 1,000 nt removed, contaminant scaffolds removed) for any of the contigs / scaffolds that were assemblies of the mitochondrial genome, rather than the nuclear genome using NCBI's BLAST+ version 2.4.0 tool BLASTN (Altschul et al. 1997; Camacho et al. 2009) with default parameters other than "-outfmt 6".

1.21.2 We annotated the scaffold using the MITOS WebServer version 806 (Bernt et al. 2013) and specifying "genetic code = 02 - Vertebrate" with default settings otherwise.

1.22 Sex identification

1.22.1 We searched NSO-wgs-v1 for matches to *S. varia CHD1W* and *CHD1Z* nucelotide sequences using NCBI's BLAST+ version 2.4.0 tool BLASTN (Altschul et al. 1997; Camacho et al. 2009) with default parameters other than "-outfmt 6".

1.22.2 We used the Geneious version 9.1.4 aligner through the "map to reference" function (Kearse et al. 2012; Biomatters 2016a) with default options to align primers 2550F and 2718R (Fridolfsson & Ellegren 1999) to the scaffolds and then extract the region bounded by the aligned primers.

1.23 Repeat annotation

1.23.1 We performed a homology-based repeat annotation of the genome assembly using RepeatMasker version 4.0.5 (Smit et al. 2013), which employs the repeat databases of the DFAM library version 1.3 (Wheeler et al. 2013) and the Repbase-derived RepeatMasker libraries version 20140131 (Jurka 1998, 2000; Jurka et al. 2005; Bao et al. 2015). Our installation of the RepeatMasker tool utilized NCBI's BLAST+ version 2.2.30 (Altschul et al. 1997; Camacho et al. 2009) and RMBlast version 2.2.28 (Smit et al. 2015) sequence search engines as well as the tandem repeats finder (TRF) version 4.0.7b (Benson 1999, 2012). We ran RepeatMasker with default options other than parameters "-gccalc -nolow -species aves". The purpose of this run was to produce a masked genome without masking of low complexity regions or simple repeats, which we could then use for downstream annotation steps.

1.23.2 We performed a *de novo* modeling of the repeat elements in the genome using RepeatModeler version 1.0.8 (Smit & Hubley 2015), which uses two *de novo* repeat finders, RECON version 1.08 (Bao & Eddy 2002) and RepeatScout version 1.0.5 (Price et al. 2005), as well as the tandem repeats finder (TRF) version 4.0.7b (Benson 1999,

2012), the RMBlast version 2.2.28 (Smit et al. 2015) sequence search engine, and RepeatMasker version 4.0.5 {Smit et al., 2015} with Repbase-derived RepeatMasker libraries version 20140131 (Jurka 1998, 2000; Jurka et al. 2005; Bao et al. 2015). We built a sequence database from our genome and ran RepeatModeler with default options.

1.23.3 We further masked the genome by running RepeatMasker again with the masked genome as input, using the repeat database created by our RepeatModeler run, and with default options other than parameters "-gccalc -nolow”.

1.23.4 We performed homology-based repeat masking using RepeatMasker as above with default options other than parameters "-gccalc -species aves”. We then performed a second run of RepeatMasker using the repeat database created by our RepeatModeler run with the masked genome as input and using default options other than parameters "-gccalc -nolow”. Our output was a second twice-masked genome with masked low complexity regions and simple repeats.

1.24 Gene annotation

1.24.1 We used the MAKER accessory script, cegma2zff, to convert the GFF file output from our CEGMA run on the GapClosed assembly into ZFF format to use in training of the gene prediction tool Semi-HMM-based Nucleic Acid Parser (SNAP) version 2006-07-28 (Korf 2004). We used the fathom tool of the SNAP package with the parameters “-categorize 1000”, followed by fathom with the parameters “-export 1000”, then the forge element of the SNAP package, then the hmm-assembler.pl script from the SNAP package to convert the ZFF files to an HMM file, which was then the newly trained gene finder that we provided SNAP in the MAKER configuration file (Campbell et al. 2014).

1.24.2 We ran MAKER using NCBI's BLAST version 2.2.31+ (Altschul et al. 1997; Camacho et al. 2009); the sequence comparison tool, exonerate version 2.2.0 (Slater & Birney 2005) with glib version 2.46.2; and the gene prediction tool, AUGUSTUS version 3.2.1 (Keller et al. 2011) for which we specified "chicken" for the gene prediction species model. We employed default parameters for all BLAST and exonerate statistics thresholds and default parameters for all other MAKER configuration options. We used Open MPI version 1.10.2 (Gabriel et al. 2004) to run MAKER on 62 cores for 50.62 hours.

1.24.3 We combined the annotations for all of the genes using the MAKER accessory scripts "fasta_merge" and "gff3_merge" with default options.

1.24.4 We assigned putative gene functions to the MAKER annotations by first obtaining the Uniprot manually annotated and non-redundant protein sequence database Swiss-Prot UniProt release 2016_04 (Consortium 2015) on 2016 April 25 and indexing it using NCBI's BLAST version 2.2.31+ (Altschul et al. 1997; Camacho et al. 2009) tool "makeblastdb" with default parameters other than the options "-input_type fasta -dbtype prot". We then compared the combined MAKER protein fasta file to the Swiss-Prot UniProt database using the BLAST 2.2.31+ tool "blastp" with default parameters other than the options "-evalue .000001 -outfmt 6 -num_alignments 1 -seg yes -soft_masking true -lcase_masking -max_hsps 1". We then used the MAKER accessory script "maker_functional_gff" to add the protein homology data to the combined MAKER GFF3 file and the MAKER accessory script "maker_functional_fasta" to add the protein homology data to the combined MAKER protein and transcript fasta files.

1.24.5 In order to identify proteins with known functional domains, we ran InterProScan version 5.18-57.0 (Jones et al. 2014) with options “-appl PfamA -iprlookup -goterms -f tsv”, which limited searches to Pfam, a database of protein family domains, on the protein sequences generated by MAKER. We then used the MAKER accessory script “ipr_update_gff” to update the MAKER-generated GFF3 file with the results of the InterProScan run and add information on protein family domain matches.

1.24.6 We then filtered transcripts with an Annotation Edit Distance (AED) less than 1 and/or a match to a Pfam domain using the option “-s” in the script “quality_filter.pl” supplied in MAKER version 3.00.0 (Cantarel et al. 2008).

1.24.7 We used the “stat” tool of GenomeTools version 1.5.1 (Gremme et al. 2013) to calculate annotation summary statistics, including distributions of gene lengths, exon lengths, number of exons per gene, and coding DNA sequence (CDS) lengths (measured in amino acids). We also used the “stat” tool of GenomeTools with the options “-addintrons” and “-intronlengthdistri” to infer intron lengths within the annotated gene boundaries and calculate the distribution of intron lengths.

1.25 Alignment

1.25.1 We aligned each set of reads to NSO-wgs-v1-masked using bwa version 0.7.12-r1044 (Li 2013a) with default options other than parameters "bwa mem -M". We separately aligned paired-end and unpaired reads. For alignment of the paired-end data, we set the insert size to be equal to our estimates from our initial assemblies. We set the parameter "-w" to be equal to twice the standard deviation of the insert size we estimated from our initial assemblies.

1.25.2 We merged the paired-end and unpaired read alignments using the Picard version 1.104 function MergeSamFiles (http://broadinstitute.github.io/picard) and sorted them using the Picard version 1.104 function SortSam (http://broadinstitute.github.io/picard), employing default settings for both tools. We next marked duplicate reads (both PCR and optical) using the Picard version 1.104 function MarkDuplicates (http://broadinstitute.github.io/picard), employing default settings.

1.25.3 We assessed the genome coverage, duplication level, and other statistics of each read set based on the read alignments. We used the Picard version 1.141 function CollectWgsMetrics (http://broadinstitute.github.io/picard) with the bam file output by MarkDuplicates as the input file, employing default settings, except setting COUNT_UNPAIRED=True to include coverage contributed by unpaired reads when calculating the alignment statistics. The default CollectWgsMetrics settings included setting the minimum mapping quality for a read to contribute coverage as 20 and the minimum base quality for a base to contribute coverage as 20. We also ran CollectWgsMetrics with the default settings and COUNT_UNPAIRED=False to obtain the portion of the total aligned reads contributed by unpaired reads.

1.25.4 In order to obtain an estimate of the insert size of the mate pair libraries independent of the N-gaps in the scaffold sequences, we divided the scaffolds into contigs at 25 or more N's using make-contig-ref.sh from NSO-genome-scripts version 1.0.0 (Hanna & Henderson 2017) with bioawk version 1.0 (Li 2013b), GNU Awk (GAWK) version 4.0.1 (Free Software Foundation 2012), and GNU fold version 8.21 (MacKenzie 2013). We then aligned the mate pair libraries to this set of contigs using bwa version 0.7.10-r789 (Li 2013a) with default options other than parameters "bwa mem -M". For alignment of

the paired-end data, we set the insert size to be equal to our estimates from our initial assemblies. We set the parameter "-w" to be equal to twice the standard deviation of the insert size we estimated from our initial assemblies. We calculated the insert sizes for each of the three mate pair libraries from these alignments using calcInsertLen.sh from NSO-genome-scripts version 1.0.0 (Hanna & Henderson 2017) with bioawk version 1.0 (Li 2013b).

1.26 Microsatellite analysis

1.26.1 We searched the assembly for 16 pairs of microsatellite primer sequences using NCBI's BLAST+ version 2.4.0 tool BLASTN (Altschul et al. 1997; Camacho et al. 2009) with default parameters other than "-outfmt 6 –word_size 7".

1.27 Barred owl divergence

1.27.1 We used 50 ng genomic DNA to prepare a whole-genome library using a Nextera DNA Sample Preparation Kit (Illumina). After tagmentation, we cleaned the reaction with a DNA Clean & Concentrator -5 kit (Zymo Research). We amplified the reaction with 5 cycles of PCR using a KAPA Library Amplification kit (KAPA Biosystems) and then cleaned the reaction with a DNA Clean & Concentrator -5 kit (Zymo Research). We used a BluePippin (Sage Science) to select library fragments in the size range of 500-700 nt, which, after subtracting the 134 nt of adapters, corresponded to selecting an average insert size of 466 nt. We cleaned the BluePippin products with 0.6X Agencourt AMPure XP (Beckman Coulter) magnetic beads and then performed a real-time PCR (rtPCR) using a KAPA Real-Time Library Amplification Kit (KAPA Biosystems) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) to amplify the library with 8 cycles PCR. We then cleaned the PCR products with a DNA Clean & Concentrator -5 kit (Zymo

Research). We lastly assessed the library fragment size distribution with a 2100 BioAnalyzer (Agilent Technologies) and the concentration of double-stranded DNA material with a Qubit 2.0 Flurometer (Invitrogen). We combined this library with others and sequenced it on two successive runs of 150 nt paired-end sequencing using a 2-lane flow cell on a HiSeq 2500 (Illumina) in rapid mode. On the first run, we obtained sequencing data from a portion of each of the two flow cell lanes. On the second run, we obtained data from a portion of one of the two flow cell lanes. We combined all of the data from the two runs for the downstream steps.

1.27.2 We performed adapter and quality trimming of the sequence data using Trimmomatic version 0.32 (Bolger et al. 2014). We used the following options: "ILLUMINACLIP:<fasta of Illumina adapter sequences>:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:28 MINLEN:36".

1.27.3 We aligned trimmed paired and unpaired reads to NSO-wgs-v1-masked using bwa mem version 0.7.12-r1044 (Li 2013a) with default options other than parameters "bwa mem -M". We separately aligned paired-end and unpaired reads. For alignment of the paired-end reads, we set the insert size to be equal to the size estimate of the final library given by the 2100 BioAnalyzer (Agilent Technologies) minus the length of the adapters, which gave an insert size of 466 nt. Additionally, for the alignment of the paired-end reads we set the parameter "-w", the maximum insert size, equal to 1000.

1.27.4 We merged the paired-end and unpaired sequence alignments using the Picard version 1.104 function MergeSamFiles (http://broadinstitute.github.io/picard) and sorted them using the Picard version 1.104 function SortSam (http://broadinstitute.github.io/picard), employing default settings for both tools. We next marked duplicate sequences (both

PCR and optical) using the Picard version 1.104 function MarkDuplicates (http://broadinstitute.github.io/picard), employing default settings.

1.27.5 We calculated various alignment statistics using the Picard version 1.141 function CollectWgsMetrics (http://broadinstitute.github.io/picard) with the bam file output by MarkDuplicates as input and employing default settings except setting COUNT_UNPAIRED=True in order to include coverage contributed by unpaired reads in the calculation of the statistics on the aligned reads. The default CollectWgsMetrics settings include setting the minimum mapping quality for a read to contribute coverage as 20 and the minimum base quality for a base to contribute coverage as 20. We also ran CollectWgsMetrics with the default settings and COUNT_UNPAIRED=False to obtain the portion of the total aligned reads contributed by unpaired reads.

1.27.6 We used Genome Analysis Toolkit (GATK) version 3.4-46 UnifiedGenotyper (McKenna et al. 2010; DePristo et al. 2011; Van der Auwera et al. 2013) to call SNPs using the *S. occidentalis* (Sequoia) and *S. varia* (CNHM<USA-OH>:ORNITH:B41533) bwa-aligned, sorted, duplicate-marked bam files as simultaneous inputs and employing default options other than setting "--output_mode EMIT_ALL_SITES".

1.27.7 We first filtered the variant file using the following GNU Awk (GAWK) version 4.0.1 (Free Software Foundation 2012) command: “awk 'NF==11 && substr($1, 1, 2) != "##" && $6>=50 && $1 != "#CHROM" && $1 != "C7961234" && $1 != "C7963448" && $1 != "C7970814" && $1 != "C8091874" && $1 != "scaffold3674"' | awk '$4=="A" || $4=="C" || $4=="G" || $4=="T"' | awk '$5=="A" || $5=="C" || $5=="G" || $5=="T"' > filtered1.vcf”. This removed lines without 11 fields, header lines, variant sites where the

Phred-scaled probability that a polymorphism exists was < 50, contaminant scaffolds, the mitochondrial genome scaffold, indels, and non-polymorphic sites.

1.27.8 We then calculated the unfiltered allele depth (the number of reads that supported an allele) summed across all of the alleles at each of the remaining variant sites using the following GNU cut version 8.21 (Ihnat et al. 2013) and GNU Awk (GAWK) version 4.0.1 (Free Software Foundation 2012) command: “cat filtered1.vcf | cut -f10,11 | awk 'BEGIN {cov} {split($1,a,":"); split(a[2],acov,","); split($2,b,":"); split(b[2],bcov,","); totcov = acov[1]+acov[2]+bcov[1]+bcov[2]; print totcov}' > vcf-coverage.out”. We then graphed these depths and calculated the mean and standard deviation (σ) of the distribution using vcf-coverage-calc.py from NSO-genome-scripts version 1.0.0 (Hanna & Henderson 2017) with Python version 2.7.12 (Python Software Foundation 2016), matplotlib version 1.5.1 (Hunter 2007; Matplotlib Development Team 2016), and NumPy version 1.11.1 (NumPy Developers 2016).

1.27.9 When calculating the nucleotide diversity both within and between samples (H_w and H_b), we removed variants where the unfiltered allele depth summed across all of the alleles was greater than 5σ greater than the mean depth, variants without information for both samples, and variants where the *S. o. caurina* genotype was homozygous for the non-reference allele. We used calc-pi-exclude-onlySPOW.sh and calc-pi-exclude-onlyBADO.sh from NSO-genome-scripts version 1.0.0 (Hanna & Henderson 2017) with GNU cut version 8.21 (Ihnat et al. 2013) and GNU Awk (GAWK) version 4.0.1 (Free Software Foundation 2012) to calculate the H_w for *S. o. caurina* and *S. varia*, respectively. We used calc-pi-exclude.sh from NSO-genome-scripts version 1.0.0 (Hanna & Henderson 2017) with GNU Awk (GAWK) version 4.0.1 (Free Software Foundation

2012) to calculate H_b for *S. o. caurina* and *S. varia*. In order to report H_w and H_b in terms of the number of nucleotide differences per site within the sample, we divided the output from the scripts above by the number of ACGT characters in NSO-wgs-v1-nuc (the whole-genome assembly without the contaminant or mitochondrial scaffolds), which we obtained using “assemblathon-stats-ex.pl” from NSO-genome-scripts (Bradnam et al. 2013; Hanna & Henderson 2017).

1.27.10 We averaged the values of H_w for *S. o. caurina* and *S. varia* and then used this average along with H_b in equation 3 from a study by Hudson, Slatkin & Maddison (1992) in order to estimate F_{ST} between *S. o. caurina* and *S. varia*.

1.28 PSMC analysis

1.28.1 In order to prepare our data for input into an analysis using an implementation of the pairwise sequentially Markovian coalescent model, PSMC version 0.6.5-r67 (Li 2015; Li & Durbin 2011), we used Samtools version 1.3.1 with HTSlib 1.3.1 (Li, Handsaker, Marshall, et al. 2016; Li et al. 2009), bcftools version 1.3.1 (Li, Handsaker, Danecek, et al. 2016), and the vcfutils.pl script from bcftools to call variants with the command “samtools mpileup -C50 –uf reference-genome.fa alignment-file.bam | bcftools call -c - | vcfutils.pl vcf2fq -d minimum-read-depth -D maximum-read-depth | gzip >variants.fq.gz”. As per the recommendation of the PSMC documentation (https://github.com/lh3/psmc), we used a third of the average read depth as the minimum read depth (-d) and twice the average read depth as the maximum read depth (-D) (-d 20 -D 126 and -d5 -D 33 for *S. o. caurina* and *S. varia*, respectively). We determined the average read depth using Samtools version 1.3.1 with HTSlib 1.3.1 (Li, Handsaker, Marshall, et al. 2016; Li et al. 2009) and GNU Awk (GAWK) version 4.0.1 (Free

Software Foundation 2012) with the command “samtools depth alignment-file.bam | awk '{sum += $3} END {print sum / NR}'”.

1.28.2 After variant calling, we used the PSMC script “fq2psmcfa” next with the command “fq2psmcfa -q20 variants.fq.gz >variants.psmcfa”. We then ran PSMC with the command “psmc -N25 -t15 -r5 -p "4+25*2+4+6" -o variants.psmc variants.psmcfa”. We next ran the PSMC scripts “psmc2history.pl” and “history2ms.pl” with the command “psmc2history.pl variants.psmc | history2ms.pl > variants.psmc_ms-cmd.sh”.

1.28.3 We ran 100 rounds of bootstraping by first splitting long reference sequences into shorter lengths in the variants.psmcfa file using the PSMC script “splitfa” with the command “splitfa variants.psmcfa >variants-split.psmcfa” and then running PSMC with the command “parallel -j25 ‘psmc -N25 -t15 -r5 -b -p "4+25*2+4+6" -o variants-split-round-{}.psmc variants-split.psmcfa’ :::: <(seq 100)”.

1.28.4 We graphed the output of our PSMC run and rounds of bootstrapping by first combining using GNU cat version 8.21 (Granlund & Stallman 2013) with the command “cat variants.psmc variants-split-round-*.psmc >variants-combined.psmc”. We then plotted the output using the PSMC script “psmc_plot.pl” with the command “psmc_plot.pl -u 4.6e-09 -g 2 variants-combined-plot variants-combined.psmc”. We used 2 years as the generation time (-g option for psmc_plot.pl) for both *S. o. caurina* and *S. varia* (Gutiérrez et al. 1995; Mazur & James 2000) although *S. o. caurina* may breed in its first year (Hamer et al. 1994) and some researchers have estimated the generation time *S. o. caurina* as 10 years (Noon & Biles 1990; U.S. Forest Service 1992). We used 4.6×10^{-9} mutations per site per generation (Smeds et al. 2016) as the mutation rate (-u option for psmc_plot.pl).

1.29 Light-associated gene analyses

1.29.1 We searched in NSO-wgs-v1 for regions orthologous to probes for 19 genes that encode proteins with light-associated functions using Geneious version 9.1.6 (Biomatters 2016b; Kearse et al. 2012) and the included version of the NCBI BLAST+ BLASTn tool (Zhang et al. 2000) with default options. On 1-10 November, 2016, we used the web version of NCBI BLAST+ version 2.5.0 (Zhang et al. 2000) with discontiguous megablast options to align the probes against sequences in the NCBI Whole-Genome-Shotgun (WGS) contigs database limited by specifying the organism *T. alba* (taxid:56313).

1.29.2 When BLAST searches were unsuccessful, we used synteny data from Ensembl (version 86; (Yates et al. 2016) to search for evidence of whole gene deletion. We identified genes flanking the gene of interest in related taxa, and subsequently used BLAST to align the reference sequences for these genes against the *S. o. caurina* and *T. alba* genome assemblies. We imported the *S. o. caurina* genome assembly into Geneious version 9.1.6 (Biomatters 2016b; Kearse et al. 2012) and used the included version of the NCBI BLAST+ BLASTn tool (Zhang et al. 2000) to search for the flanking genes in our assembly. We used the web version of NCBI BLAST+ version 2.5.0 (Zhang et al. 2000) to align the flanking genes against *T. alba* sequences in the NCBI Whole-Genome-Shotgun (WGS) contigs database.

1.29.3 We used the NCBI BLAST+ version 2.5.0 blastn tool (Zhang et al. 2000) with the discontiguous megablast option to align a reference *Opn4m* sequence to fifteen avian retinal transcriptomes in NCBI's Sequence Read Archive (SRA) (Leinonen et al. 2011; NCBI Resource Coordinators 2016) including the pied harrier (*Circus melanoleucos*) (SRA accession SRR3203217), long-eared owl (*Asio otus*) (SRA accession

SRR3203220), eastern grass owl (*Tyto longimembris*) (SRA accession SRR3203222), hoopoe (*Upupa epops*) (SRA accession SRR3203224), Eurasian eagle-owl (*Bubo bubo*) (SRA accession SRR3203225), black-winged kite (*Elanus caeruleus*) (SRA accession SRR3203227), Eurasian scops owl (*Otus scops*) (SRA accession SRR3203230), common kestrel (*Falco tinnunculus*) (SRA accession SRR3203231), grey-faced buzzard (*Butastur indicus*) (SRA accession SRR3203233), besra (*Accipiter virgatus*) (SRA accession SRR3203234), cinereous vulture (*Aegypius monachus*) (SRA accession SRR3203236), Eurasian hobby (*Falco subbuteo*) (SRA accession SRR3203238), grey-headed woodpecker (*Picus canus*) (SRA accession SRR3203240), little owl (*Athene noctua*) (SRA accession SRR3203242), Indian scops owl (*Otus bakkamoena*) (SRA accession SRR3203243) (Wu et al. 2016).

2 Supplementary Results and Discussion

2.1 Scaffold numbering

2.1.1 When referring to specific scaffolds in the results and discussion sections, we have inserted a dash ("-") between the word "scaffold" and the scaffold number for legibility. These dashes are not present in any of the assembly data files. Thus, "scaffold-1085" referenced in the manuscript will appear as "scaffold1085" in the assembly and other associated files.

3 Supplementary Tables

Table S1. Sequence data collected for use in genome assembly. We here provide information on the insert size, fragmentation method, amplification, sequencing length, and raw data quantity for all libraries sequenced for this genome assembly. We have numbered the libraries and refer to these numbers in other sections of this manuscript.

Library number	Library name	Average insert size (nt)	Insert size standard deviation (nt)	Library Fragmentation method	PCR amplification used (Yes / No)	Paired-end read lengths forward / reverse (nt)	Raw reads passing onboard Illumina quality filter coverage of 1.5 Gnt genome (1X-fold coverage)
1	Nextera350nt lane 1	247	118	Nextera	Yes	100 / 100	9.80
2	Nextera350nt lane 2	247	118	Nextera	Yes	100 / 100	26.44
3	Hydroshear	500	52	Hydroshear	Yes	350 / 250	2.55
4	Nextera550nt lane 1	560	25	Nextera	Yes	300 / 300	3.65
5	Nextera550nt lane 2	560	25	Nextera	Yes	375 / 225	8.90
6	Nextera700nt	566	194	Nextera	Yes	150 / 150	31.14
7	noPCR550nt	619	132	Covaris	No	350 / 250	3.50
8	PCR900nt	687	58	Covaris	Yes	350 / 250	2.04
9	MP4kb	3,316	213	Nextera Mate Pair	Yes	100 / 100	7.84
10	MP7kb	5,904	537	Nextera Mate Pair	Yes	100 / 100	8.48
11	MP11kb	9,615	1930	Nextera Mate Pair	Yes	100 / 100	8.19

Table S2. Preliminary assembly parameters. We here report the parameters used in our preliminary assemblies using SOAPdenovo2. "Trim level" indicates the average Phred score to which we trimmed using Trimmomatic. A higher Phred score indicates a more restrictive trimming. "Error correction" refers to whether we performed error correction on the input reads for the assembly. We provide information on how we specified that the assembler use the paired-end and unpaired data for each assembly. For a given assembly, we note which libraries provided data and in which portions of the assembly process that data was used. For a given portion of the assembly process, we give the numbers of the utilized libraries followed, in parentheses, by the rank given to each library in the assembly configuration file. Please refer to Table S1 for information about the libraries to which the numbers refer. An asterisk is next to the preliminary assembly that we chose to use as the basis for the final assembly.

Assembly	Trim level	Error correction	Assembly notes	Unpaired data - only contig	Paired-end data - only scaffold	Paired-end data - both contig and scaffold	Unpaired data - only gap closure
1	28	No	N/A	1-11 (6)	9 (3), 10 (4), 11 (5)	1-3 (1), 6 (2), 7 (1), 8 (2)	None
2	28	No	N/A	1-2 (6), 4-11 (6)	9 (3), 10 (4), 11 (5)	1-2 (1), 6 (2), 7 (1), 8 (2)	None
3	28		Only reads merged with BBMerge used as unpaired data	1-5 (6), 7-8 (6)	9 (3), 10 (4), 11 (5)	1-2 (1), 6 (2), 7 (1), 8 (2)	None
4*	17	Yes	N/A	1-2 (6), 4-11 (6)	9 (3), 10 (4), 11 (5)	1-2 (1), 6 (2), 7 (1), 8 (2)	None
5	28	No	No merging of paired-end reads performed		9 (3), 10 (4), 11 (5)	1-5 (1), 6 (2), 7 (1), 8 (2)	None
6	28	No	N/A	1-11 (6)	9 (3), 10 (4), 11 (5)	1-3 (1), 6 (2), 7 (1)	None
7	28	No	N/A	1-11 (6)	9 (3), 10 (4), 11 (5)	1 (1), 2 (1), 3 (1), 4 (1), 5 (1), 6 (2), 7 (1), 8 (2)	None
8	28	No	No merging of paired-end reads performed.		9 (3), 10 (4), 11 (5)	1 (1), 2 (1), 4 (1), 5 (1), 6 (2), 7 (1), 8 (2)	None
9	28	No	Only reads merged with BBMerge used as unpaired data	1-2 (6), 4-5 (6), 7-8 (6)	9 (3), 10 (4), 11 (5)	1 (1), 2 (1), 6 (2), 7 (1), 8 (2)	None
10	28	No	Only reads merged with BBMerge used as unpaired data	1-2 (6), 4-5 (6), 7-8 (6)	9 (3), 10 (4), 11 (5)	1-2 (1), 4-5 (1), 6 (2), 7 (1), 8 (2)	None
11	17	Yes	Only reads merged with BBMerge used as unpaired data, library 3 excluded.	1-2 (6), 4-5 (6), 7-8 (6)	9 (3), 10 (4), 11 (5)	1-2 (1), 4-5 (1), 6 (2), 7 (1), 8 (2)	None
12	17	Yes	All unpaired reads used, library 3 excluded.	1-2 (6) 4-5 (6), 6-11 (6)	9 (3), 10 (4), 11 (5)	1-2 (1), 4-5 (1), 6 (2), 7 (1), 8 (2)	None
13	17	Yes	Reads merged with BBMerged used for contig assembly, other unpaired reads used only for gap closure.	1-2 (6), 4-5 (6), 7-8 (6)	9 (3), 10 (4), 11 (5)	1-2 (1), 2 (1), 4-5 (1), 6 (2), 7 (1), 8 (2)	1-2 (7), 4-11 (7)
14	7	Yes	N/A	1-2 (6), 4-11 (6)	9 (3), 10 (4), 11 (5)	1-2 (1), 4-5 (1), 6 (2), 7 (1), 8 (2)	None

Table S3. Light-associated gene searches information. This table provides details on the reference sequences used for and the results of our searches for light-associated genes in the genome assemblies of *Strix occidentalis caurina* and *Tyto alba*. "Stop" indicates the presence of a premature stop codon. "Del" indicates a frameshift deletion. "Ins" indicates a frameshift insertion.

Gene	**Reference Sequence**	***Strix occidentalis* Sequence**	***Tyto alba* Sequence**
SWS1	GenBank: AH007798 *Columba livia*	**No BLAST results**	**No BLAST results**
SWS1 notes	**Synteny:** *Taeniopygia guttata* and *Homo sapiens,* 5' end *FLNC* (REV), 3' end *CALU* (REV); *Anolis carolinensis*, 3' end *CALU* (REV)	*FLNC:* scaffold-4221 *CALU:* scaffold-15	No gene predictions for *FLNC* or *CALU* in *Tyto*
SWS2	GenBank: AH007799 *Columba livia*	scaffold-4153 & scaffold-7110: Functional	**No BLAST results**
SWS2 notes	**Synteny:** *Anolis carolinensis* and *Xenopus laevis* 5' end *MECP2* (REV), 3' end *LWS;* avian contigs in Ensembl are very short and do not include flanking genes	Only exons 1 (partial), 2 and 3 recovered; partial exon 1 flanked by N's, and exon 3 is towards the end of the scaffold; 2 different scaffolds; 100% identical except 1-nt diff in exon 3, nonsynonymous	*MECP2* and *LWS* not predicted in *Tyto*
Rh1	GenBank: AH007730 *Columba livia*	scaffold-133: Functional	JJRD01003728, JJRD01003729: Functional
Rh2	GenBank: AH007731 *Columba livia*	scaffold-1932: Functional	**JJRD01131248, JJRD01131249: Pseudogene (exon 1: 29-nt del; exon 2: stop; exon 3: stop; exon 4: 2-nt del)**
LWS	GenBank: AH007800 *Columba livia*	scaffold-6263: Functional	**No BLAST results**
LWS notes	**Synteny:** *Anolis carolinensis* 5' end *SWS2,* 3' end TEX28 (REV); *Xenopus laevis* 5' end *SWS2*, 3' end *AVPR2*; avian contigs in Ensembl are very short and do not include flanking genes	Only exons 2, partial 5 and 6; 3-5 are N's, no hits for exon 1	No gene predictions for *SWS2, AVPR2* or *TEX28*
OpnP	GenBank: U15762, WGS: AADN03007691 *Gallus gallus*	**No BLAST results; After BLASTing intergenic region, has hit with *Gallus gallus* genomic pinopsin, non-coding region 5' of cds is retained**	**JJRD01162372, JJRD01162373: Pseudogene (exon 1: start codon mutation ACA, 13-nt del, 2-nt ins, 1-nt del, exon 2: 1-nt del; intron 3-exon 4 boundary: 21 nt-del; exon 4: 7-nt del, 2-nt del; exon 5: 1-nt del)**
OpnP notes	**Synteny:** *Gallus gallus DOC2B,* 5' end, 3' end *TEX14* (REV); *Ficedula albicollis DOC2B*, 5' end	DOC2B: scaffold-86 TEX14: scaffold-86	
OpnVA	GenBank: EF055883, WGS: AADN03005037 *Gallus gallus*	Scaffold205: Functional	JJRD01088850, JJRD01088852, JJRD01106859, JJRD01168068: Functional

Gene	**Reference Sequence**	***Strix occidentalis* Sequence**	***Tyto alba* Sequence**
Opn4x	GenBank: NM_204625, WGS: AADN03004364 *Gallus gallus*	scaffold-147: Functional	JJRD01038044: Functional
Opn4m	GenBank: AY882944, WGS: AADN04000143 *Gallus gallus*	**scaffold-219: Pseudogene? (exon 8: stop, 4-nt del)**	**JJRD01098086, JJRD01098087: Pseudogene? (exon 8: 4-nt del; intron 11: splice donor mutation GT to AT)**
Opn3	GenBank: XM_426139, WGS: AADN04000318 *Gallus gallus*	scaffold-728: Functional	JJRD01072701: Functional (No BLAST results for exon 1)
Opn5	GenBank: NM_001130743 WGS: AADN04000287 *Gallus gallus*	scaffold-546: Functional	JJRD01001581, JJRD01133804: Functional
Opn5L1	GenBank: NM_001310056, WGS: AADN04000228 *Gallus gallus*	scaffold-6: Functional	JJRD01004196: Functional
Opn5L2	GenBank: NM_001162892, WGS: AADN04000287 *Gallus gallus*	scaffold-722: Functional	JJRD01082691: Functional
RRH	GenBank: NM_001079759, WGS: AADN04000018 *Gallus gallus*	scaffold-22: Functional	JJRD01123735: Functional
RGR	GenBank: NM_001031216, WGS: AADN04000143 *Gallus gallus*	scaffold-219: Functional	JJRD01065549: Functional
EEVS-like	GenBank: XM_013180282, WGS: AOGC01018216 *Anser cygnoides*	scaffold-133: Functional	JJRD01160345: Functional
MT-Ox	GenBank: XM_015293238, WGS: AADN04000009 *Gallus gallus*	scaffold-133: Functional	JJRD01160345, JJRD01160346, JJRD01160347, JJRD01160348: Functional
Photolyase	GenBank: XM_422729, WGS: AADN04000078 *Gallus gallus*	scaffold-742: Functional	JJRD01136093, JJRD01136094: Functional
CYP2J19	GenBank: XM_422553, WGS: AADN04000032 *Gallus gallus*	**scaffold-313: Pseudogene? (exon 9: 1-nt ins, 2-nt del)**	**JJRD01034859: Pseudogene (exon 1: stop; exon 3: 5-nt del; exon 5: stop; exon 6: stop)**

Table S4. Assembly metrics with a range of cutoffs. These are statistics on the final (post gap closing) assembly that display the consequence of choosing various cutoffs for minimum scaffold length and the number of N's that separate a contig. We have marked the line with the cutoffs and statistics that correspond to the final chosen assembly version with an asterisk.

Scaffold minimum length (nt)	Scaffold N50 (nt)	Scaffold L50	Number of Scaffolds	Total sequence length (nt)	Number of N's to split contigs	Contig N50	Contig L50	Number of contigs	Total sequence length (nt)
1000*	3,983,020	92	8,113	1,255,568,683	25	171,882	2,057	27,258	1,241,846,690
1000	-	-	-	-	20	167,327	2,112	27,729	1,241,836,309
1000	-	-	-	-	15	163,476	2,166	28,200	1,241,828,287
1000	-	-	-	-	10	159,062	2,233	28,719	1,241,822,133
1000	-	-	-	-	5	155,200	2,286	29,229	1,241,818,593
1000	-	-	-	-	1	51,301	7,054	65,092	1,241,782,051
500	3,937,821	93	17,952	1,262,291,236	25	170,589	2,076	37,544	1,248,502,317
500	-	-	-	-	20	166,062	2,132	38,023	1,248,491,764
500	-	-	-	-	15	162,595	2,186	38,504	1,248,483,572
500	-	-	-	-	10	158,193	2,254	39,038	1,248,477,239
500	-	-	-	-	5	153,747	2,308	39,562	1,248,473,599
500	-	-	-	-	1	50,930	7,119	76,379	1,248,436,081
300	3,915,799	95	48356	1,273,290,518	25	168,721	2,109	67,949	1,259,501,544
300	-	-	-	-	20	164,817	2,166	68,428	1,259,490,991
300	-	-	-	-	15	161,269	2,220	68,909	1,259,482,799
300	-	-	-	-	10	156,434	2,289	69,443	1,259,476,466
300	-	-	-	-	5	152,072	2,344	69,967	1,259,472,826
300	-	-	-	-	1	50,425	7,228	106,823	1,259,435,266
None	1,836,279	209	3,754,965	1,882,109,172	25	81,400	4,678	3,774,558	1,868,320,198
None	-	-	-	-	20	79,089	4,800	3,775,037	1,868,309,645
None	-	-	-	-	15	77,624	4,921	3,775,518	1,868,301,453
None	-	-	-	-	10	76,045	5,061	3,776,052	1,868,295,120
None	-	-	-	-	5	73,935	5,180	3,776,576	1,868,291,480
None	-	-	-	-	1	25,761	15,609	3,813,432	1,868,253,920

Table S5. Final SOAPdenovo2 parameters. This table lists the SOAPdenovo2 parameters that we specified for each library to generate the final assembly.

Library	Paired or unpaired reads	Configuration file insert size (nt)	Used in contig or scaffold building	Assembly usage rank	Pair number cutoff	Mapping length (nt)
Nextera350nt lane 1	paired	247	both	1	3	32
Nextera350nt lane 2	paired	247	both	1	3	32
Nextera700nt	paired	566	both	2	3	32
noPCR550nt	paired	619	both	1	3	32
PCR900nt	paired	687	both	2	3	32
MP4kb	paired	3,316	scaffold	3	5	35
MP7kb	paired	5,904	scaffold	4	5	35
MP11kb	paired	9,615	scaffold	5	5	35
Nextera350nt lane 1	unpaired	N/A	contig	6	3	32
Nextera350nt lane 2	unpaired	N/A	contig	6	3	32
Nextera550nt lane 1	unpaired	N/A	contig	6	3	32
Nextera550nt lane 2	unpaired	N/A	contig	6	3	32
Nextera700nt	unpaired	N/A	contig	6	3	32
noPCR550nt	unpaired	N/A	contig	6	3	32
PCR900nt	unpaired	N/A	contig	6	3	32
MP4kb	unpaired	N/A	contig	6	3	32
MP7kb	unpaired	N/A	contig	6	3	32
MP11kb	unpaired	N/A	contig	6	3	32

Table S6. Full assembly metrics. Listed here are metrics on the full assembly (no contaminate or mitochondrial sequences removed) before gap-closing, after gap-closing, and after gap-closing and removal of all contigs and scaffolds less than 1000 nt in length. Strings of 25 or more N's broke scaffolds into contigs.

Assembly version	**No gap-closing, scaffolds and contigs <1000 nt removed**	**Gap-closed, no scaffolds or contigs removed**	**Gap-closed, scaffolds and contigs <1000 nt removed**
Number of scaffolds	3,754,965	3,754,965	8,113
Total size of scaffolds	1,884,424,465 nt	1,882,109,172 nt	1,255,568,683 nt
Longest scaffold	15,783,852 nt	15,750,186 nt	15,750,186 nt
Shortest scaffold	128 nt	128 nt	1,000 nt
Number of scaffolds > 1K nt	8,117 (0.2%)	8,100 (0.2%)	8,100 (99.8%)
Number of scaffolds > 10K nt	1,755 (0.0%)	1,747 (0.0%)	1,747 (21.5%)
Number of scaffolds > 100K nt	661 (0.0%)	661 (0.0%)	661 (8.1%)
Number of scaffolds > 1M nt	303 (0.0%)	303 (0.0%)	303 (3.7%)
Number of scaffolds > 10M nt	9 (0.0%)	9 (0.0%)	9 (0.1%)
Mean scaffold size	502 nt	501 nt	154,760 nt
Median scaffold size	150 nt	150 nt	1,903 nt
N50 scaffold length (L50 scaffold count)	1,843,286 nt (209)	1,836,279 nt (209)	3,983,020 nt (92)
N60 scaffold length (L60 scaffold count)	622,124 nt (370)	619,581 nt (371)	3,012,707 nt (129)
N70 scaffold length (L70 scaffold count)	255 nt (216,224)	255 nt (218,948)	2,142,451 nt (178)
N80 scaffold length (L80 scaffold count)	174 nt (1,110,557)	174 nt (1,113,218)	1,545,070 nt (246)
N90 scaffold length (L90 scaffold count)	143 nt (2,336,944)	143 nt (2,338,563)	618,731 nt (372)
scaffold %GC	42.81%	43.82%	41.31%
scaffold %N	2.89%	0.74%	1.10%
Percentage of assembly in scaffolded contigs	66.4%	65.7%	98.5%
Percentage of assembly in unscaffolded contigs	33.6%	34.3%	1.5%
Average number of contigs per scaffold	1.0	1.0	3.4
Average length of break (>25 Ns) between contigs in scaffold	311	703	716
Number of contigs	3,929,051	3,774,558	27,258
Number of contigs in scaffolds	179,957	22,374	21,480
Number of contigs not in scaffolds	3,749,094	3,752,184	5,778
Total size of contigs	1,830,129,061 nt	1,868,320,198 nt	1,241,846,690 nt
Longest contig	186,255 nt	1,259,046 nt	1,259,046 nt
Shortest contig	5 nt	128 nt	130 nt
Number of contigs > 1K nt	123,899 (3.2%)	23,921 (0.6%)	23,921 (87.8%)
Number of contigs > 10K nt	37,347 (1.0%)	12,374 (0.3%)	12,374 (45.4%)
Number of contigs > 100K nt	58 (0.0%)	3,909 (0.1%)	3,909 (14.3%)
Number of contigs > 1M nt	0 (0.0%)	8 (0.0%)	8 (0.0%)
Mean contig size	466 nt	495 nt	45,559 nt
Median contig size	150 nt	150 nt	6,696 nt
N50 contig length (L50 contig count)	7,855 nt (46,857)	81,400 nt (4,678)	171,882 nt (2,057)
N60 contig length (L60 contig count)	3,275 nt (81,604)	33521 nt (8,121)	134,419 nt (2,876)
N70 contig length (L70 contig count)	254 nt (448,713)	255 nt (254,707)	98,599 nt (3,956)
N80 contig length (L80 contig count)	170 nt (1,346,253)	173 nt (1,148,670)	66,629 nt (5,485)
N90 contig length (L90 contig count)	142 nt (2,548,885)	142 nt (2,367,834)	34,559 nt (8,023)

Table S7. Statistics from after quality-filtering MAKER annotations. This is a table of annotation summary statistics resulting from quality-filtering our MAKER pipeline annotation output.

	Values post -s filter
parsed genome node DAGs	745,622
sequence regions	8,112 (total length: 1,255,013,157 nt)
multi-features	15,712
genes	16,718
protein-coding genes	16,718
mRNAs	16,718
protein-coding mRNAs	16,718
exons	146,689
CDSs	146,217

Table S8. Mitochondrial genome assembly gene annotations. This is a table of the gene annotations of the assembly of a partial mitochondrial genome represented by scaffold-3674. The coordinates are 1-based.

Gene	Scaffold	Start position	End position	Direction
tRNA^{Thr}	scaffold3674	231	299	-
Cytb	scaffold3674	307	1431	-
ND5	scaffold3674	1463	3268	-
tRNA^{Leu1}	scaffold3674	3269	3339	-
tRNA^{Ser1}	scaffold3674	3342	3407	-
tRNA^{His}	scaffold3674	3410	3479	-
ND4L	scaffold3674	3490	4857	-
ND4L	scaffold3674	4854	5147	-
tRNA^{Arg}	scaffold3674	5149	5218	-
ND3_b	scaffold3674	5224	5397	-
ND3_a	scaffold3674	5399	5572	-
tRNA^{Gly}	scaffold3674	5573	5641	-
COIII	scaffold3674	5643	6425	-
ATP6	scaffold3674	6431	7108	-
ATP8	scaffold3674	7105	7266	-
tRNA^{Lys}	scaffold3674	7268	7338	-
COII	scaffold3674	7357	8031	-
tRNA^{Asp}	scaffold3674	8034	8102	-
tRNA^{Ser2}	scaffold3674	8106	8177	+
COI	scaffold3674	8178	9710	-
tRNA^{Tyr}	scaffold3674	9721	9791	+
tRNA^{Cys}	scaffold3674	9792	9860	+
tRNA^{Asn}	scaffold3674	9863	9936	+
tRNA^{Ala}	scaffold3674	9938	10006	+
tRNA^{Trp}	scaffold3674	10008	10083	-
ND2	scaffold3674	10094	11122	-
tRNA^{Met}	scaffold3674	11123	11191	-
tRNA^{Gln}	scaffold3674	11191	11261	+
tRNA^{Ile}	scaffold3674	11273	11344	-
ND1	scaffold3674	11352	12299	-
tRNA^{Leu2}	scaffold3674	12314	12387	-
16S	scaffold3674	12387	13982	-
tRNA^{Val}	scaffold3674	13983	14054	-
12S	scaffold3674	14054	15041	-
tRNA^{Phe}	scaffold3674	15041	15108	-
tRNA^{Glu}	scaffold3674	21542	21614	+

Table S9. Information on searches for light-associated genes in non-owl genome assemblies. This table provides information on the results of our searches for a subset of the light-associated genes in several non-owl avian genome assemblies. "Stop" indicates the presence of a premature stop codon. "Del" indicates a frameshift deletion. For these searches we employed the same reference sequences used in the owl genome searches, detailed in Table S3.

	Rh2	***OpnP***	***Opn4m***	***CYP2J19***
Reference Sequence	GenBank: AH007731 *Columba livia*	GenBank: U15762, WGS: AADN03007691 *Gallus gallus*	GenBank: AY882944, WGS: AADN04000143 *Gallus gallus*	GenBank: XM_422553, WGS: AADN04000032 *Gallus gallus*
Aquila chrysaetos **Sequence**	JRUM01011001	JRUM01006324	**JRUM01004396: Pseudogene? (exon 9: stop)**	JRUM01002169
Cathartes aura **Sequence**	JMFT01083953	JMFT01020150, JMFT01020151, JMFT01020152, JMFT01020153	JMFT01012857, JMFT01012858, JMFT01012859	JMFT01168756
Colius striatus **Sequence**	JJRP01038063, JJRP01092220	JJRP01068983	**JJRP01099016, JJRP01099018, JJRP01099019: Pseudogene? (exon 9: 1-bp del; intron 9: splice donor mutation GT to TT; exon 11: stop)**	JJRP01092926
Leptosomus discolor **Sequence**	JJRK01095962, JJRK01095963	JJRK01016598, JJRK01016599	**JJRK01001211, JJRK01001212, JJRK01001213: Pseudogene? (intron 10: splice donor mutation GT to GA)**	JJRK01096026
Apaloderma vittatum **Sequence**	JMFV01047445, JMFV01047446	JMFV01046166, JMFV01046167	JMFV01094831	JMFV01067118, JMFV01102670, JMFV01104326, JMFV01105382
Buceros rhinoceros **Sequence**	JMFK01024225	JMFK01144445, JMFK01144446, JMFK01144447, JMFK01144448,	**JMFK01158949, JMFK01158950, JMFK01158951, JMFK01158952: Pseudogene? (exon 1: start codon mutation CTG)**	JMFK01006414, JMFK01073748
Picoides pubescens **Sequence**	JJRU01080411, JJRU01080413	JJRU01064065	JJRU01054812	JJRU01010544, JJRU01010545
Merops nubicus **Sequence**	JJRJ01051189	JJRJ01058175	JJRJ01007844	JJRJ01011917, JJRJ01033855

Table S10. Details of branch tests. This table gives the details of the branch tests performed to test for evidence of changes in selection pressure on the owl branches. "BG" indicates the background branches, "lnL" denotes the log likelihood of the model, "LRT" denotes the value of the likelihood ratio test (given by 2 times the difference in the likelihoods of the models), and "cf" denotes the codon frequency model used to calculate the equilibrium codon frequencies with "cf 1" indicating that we used the average nucleotide frequencies and "cf 2" indicating that we used the average nucleotide frequencies at each of the 3 codon positions. "Model" corresponds to the number of ω values employed among branches with one ω value assumed for all branches under model "0", two ω values used under model "1", and 3 ω values used with model "2". "*Tyto*" and "*Strix*" indicate whether the value pertains to sequence in the *Tyto alba* or *Strix occidentalis caurina* genome assembly, respectively. For model comparisons, bold font indicates significant difference ($p < 0.05$) between models.

Gene	Model	BG ω	*Tyto* ω	*Strix* ω	Stem Owl ω	lnL	Models compared	LRT
CYP2J19 **(cf 1)**	0	0.206				-5045.714		
	1	0.173	0.719			-5029.495	**1 vs. 0**	32.437
	2	0.164	0.719	0.336		-5027.178	**2 vs. 1**	4.633
CYP2J19 **(cf 2)**	0	0.194				-5050.277		
	1	0.163	0.681			-5034.027	**1 vs. 0**	32.499
	2	0.154	0.680	0.333		-5031.418	**2 vs. 1**	5.219
OPN4M **(cf 1)**	0	0.214				-3345.378		
	1	0.192	0.448	0.448	0.895	-3341.951	1 vs. 0	6.854
OPN4M **(cf 2)**	0	0.213				-3350.019		
	1	0.190	0.452	0.452	0.864	-3346.487	**1 vs. 0**	7.066
OPNP **(cf 1)**	0	0.234				-3937.560		
	1	0.180	0.695			-3918.377	**1 vs. 0**	38.446
OPNP **(cf 2)**	0	0.152				-3892.939		
	1	0.114	0.508			-3870.379	**1 vs. 0**	45.121
RH2 **(cf 1)**	0	0.079				-3155.354		
	1	0.057	0.367			-3139.086	**1 vs. 0**	32.536
	2	0.052	0.358	0.208		-3136.501	**2 vs. 1**	5.170
RH2 **(cf 2)**	0	0.043				-3054.733		
	1	0.031	0.219			-3037.200	**1 vs. 0**	35.065
	2	0.028	0.205	0.158		-3033.836	**2 vs. 1**	6.728

Table S11. Details of branch-site tests. This table provides details of the tests performed using branch-site models implemented in the phylogenetic analysis by maximum likelihood (PAML) package to detect positive selection affecting certain sites on the owl lineages. "*Tyto*" and "*Strix*" indicate whether the values pertain to sequence in the *Tyto alba* or *Strix occidentalis caurina* genome assembly, respectively. "BG" indicates the background branches, "FG" denotes the foreground branch, "lnL" denotes the log likelihood of the model, "LRT" denotes the value of the likelihood ratio test (given by 2 times the difference in the likelihoods of the models), and "cf" denotes the codon frequency model used to calculate the equilibrium codon frequencies with "cf 1" indicating that we used the average nucleotide frequencies and "cf 2" indicating that we used the average nucleotide frequencies at each of the 3 codon positions. "Site class" indicates the ω category with "0" indicating sites under purifying selection, "1" sites under relaxed selection, "2a" sites that are under positive selection on the foreground branch and under purifying selection on the background branches, and "2b" indicating positive selection on the foreground branch and relaxed selection on the background branches. "Proportion" indicates the proportion of sites in a given class. "Model" denotes either the positive selection model ("Positive") or the null model ("Null").

Gene	Taxon	Site class	Proportion	BG ω	FG ω	Model	lnL	LRT
***OPN4M* (cf 1)**	*Strix*	0	0.778	0.047	0.047			
		1	0.184	1	1			
		2a	0.031	0.047	4.291			
		2b	0.007	1	4.291			
						Positive	-3305.681	
						Null	-3305.984	-0.605
	Tyto	0	0.773	0.046	0.046			
		1	0.190	1	1			
		2a	0.030	0.046	1.660			
		2b	0.007	1	1.660			
						Positive	-3306.308	
						Null	-3306.325	-0.033
***OPN4M* (cf 2)**	*Strix*	0	0.773	0.047	0.047			
		1	0.182	1	1			
		2a	0.036	0.047	4.051			
		2b	0.009	1	4.051			
						Positive	-3310.564	
						Null	-3310.887	-0.646
	Tyto	0	0.788	0.050	0.050			
		1	0.189	1	1			
		2a	0.019	0.050	2.072			
		2b	0.004	1	2.072			
						Positive	-3311.582	
						Null	-3311.605	-0.047

4 Supplementary Figures

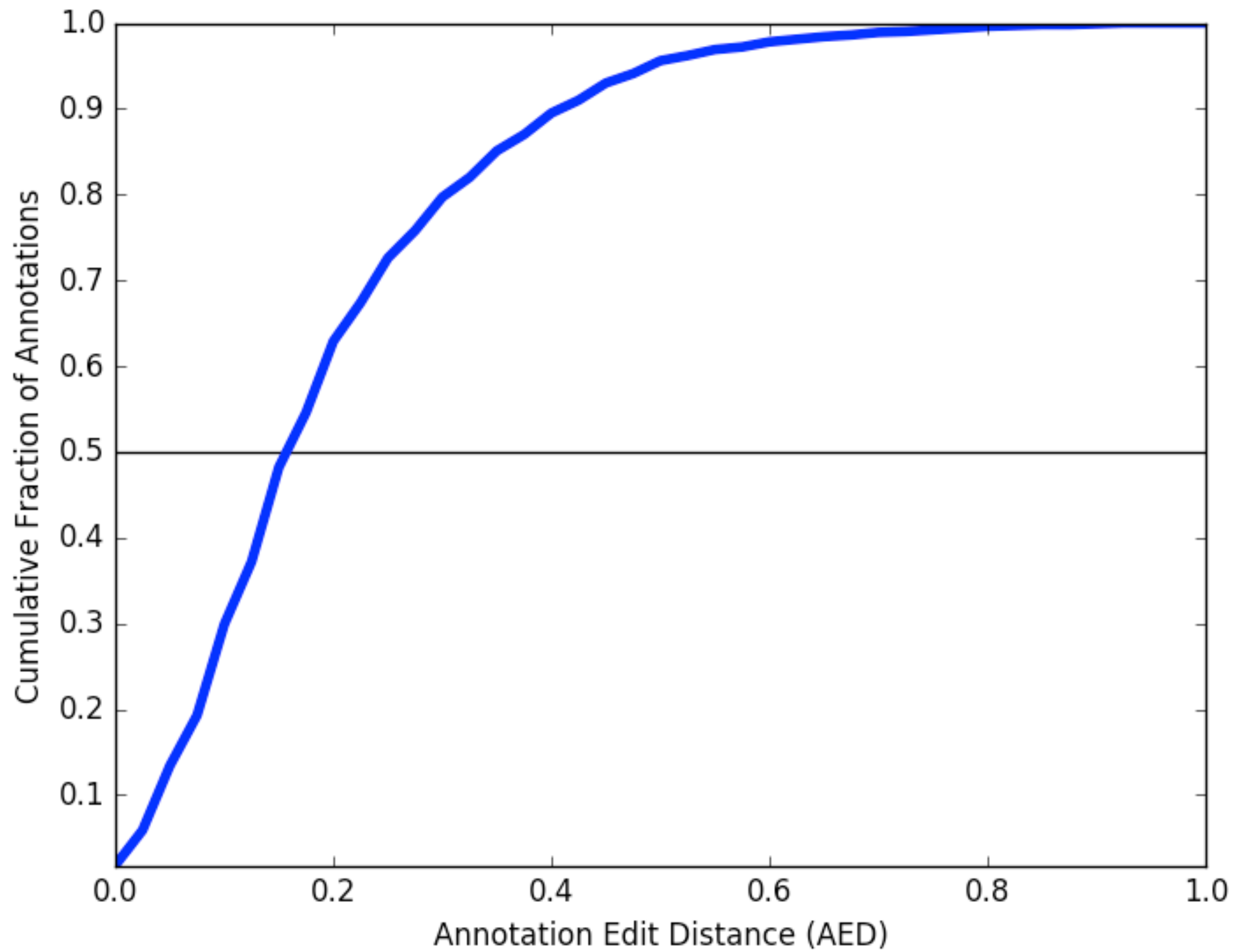

Figure S1. Cumulative distribution of annotation edit distances of MAKER-generated annotations. This is a graph of the cumulative distribution of annotation edit distances (AED) of the annotations generated by MAKER. Included here are all of the annotations in the MAKER final output. We have drawn a horizontal line denoting 50% of the annotations. After quality filtering, the cumulative distribution appeared identical.

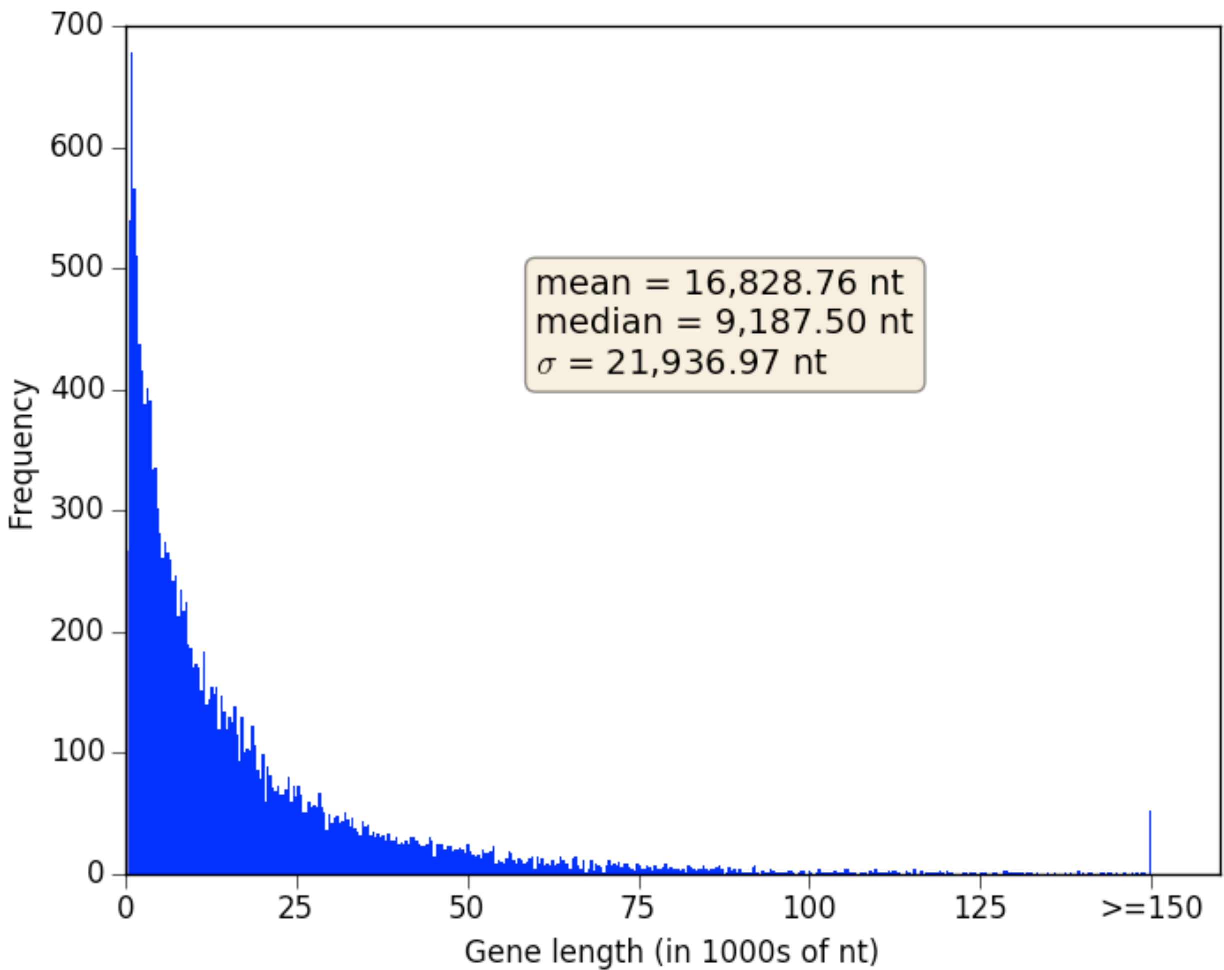

Figure S2. Histogram of the lengths of genes annotated by MAKER. This is a histogram of the distribution of the lengths of genes annotated by MAKER. We included all of the gene annotations in the MAKER final output. We grouped the values into 400 frequency bins, one of these including all genes greater than or equal to 150,000 nt in length. We have provided the mean, median, and standard deviation of the gene lengths in a text box.

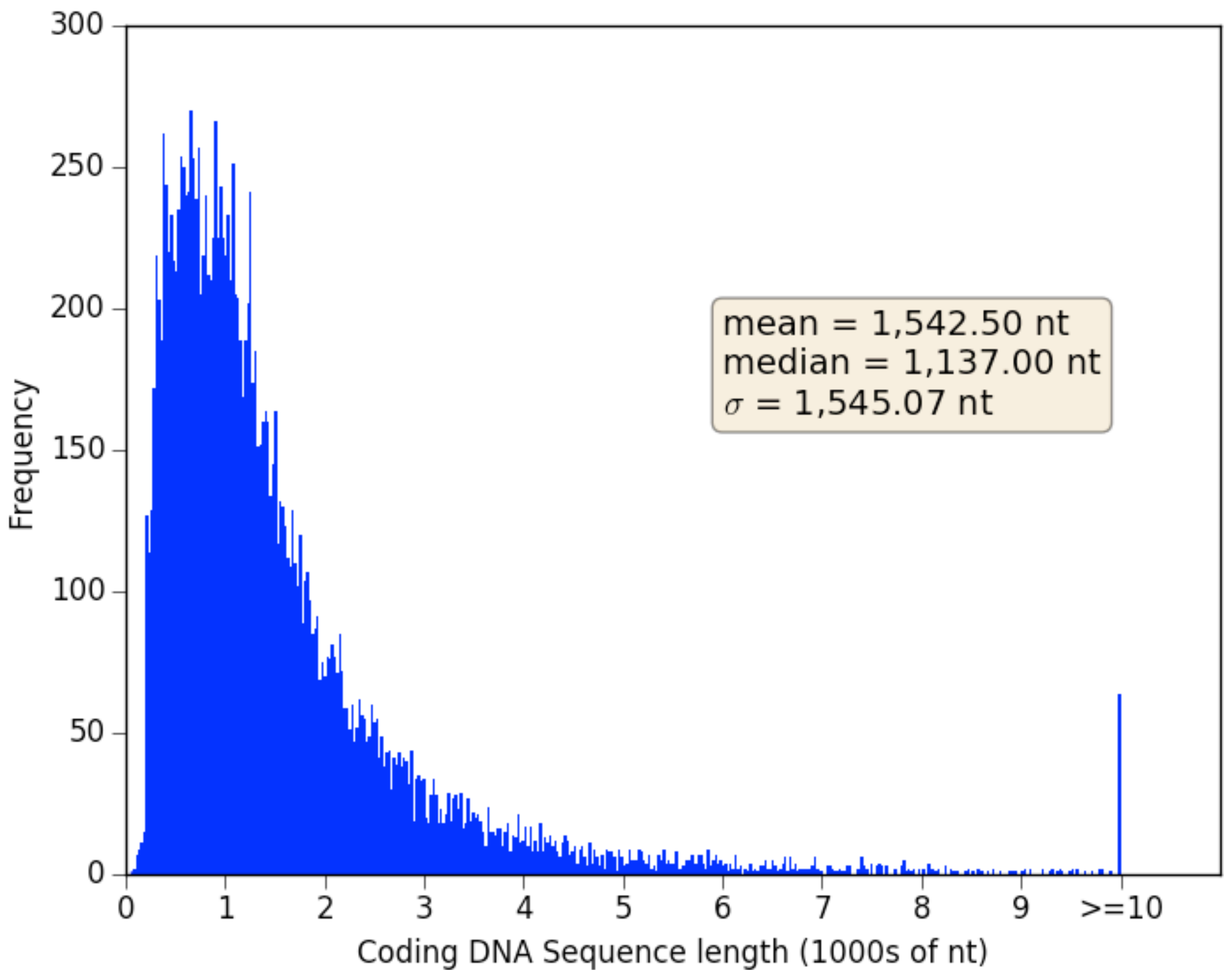

Figure S3. Histogram of the coding DNA sequence length in genes annotated by MAKER. This is a histogram of the lengths of coding DNA sequences in genes annotated by MAKER. We included all of the gene annotations in the MAKER final output. We grouped the values into 400 frequency bins, one of these including all coding DNA sequences greater than or equal to 10,000 nt in length. We have provided the mean, median, and standard deviation of the lengths in a text box.

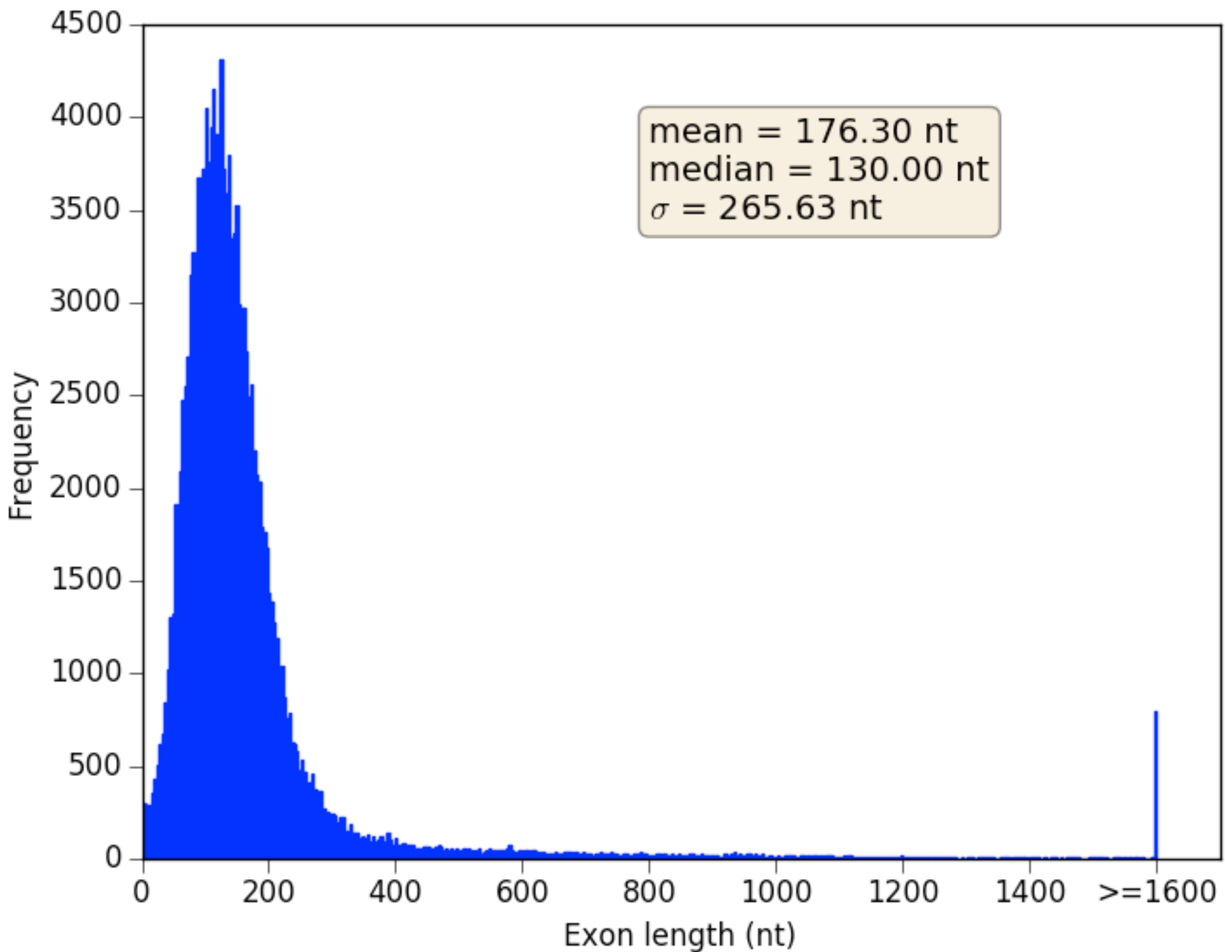

Figure S4. Histogram of the lengths of exons in genes annotated by MAKER. This is a histogram of the lengths of exons in genes annotated by MAKER. We included the exons from all of the gene annotations in the MAKER final output. We grouped the values into 400 frequency bins, one of these including all exons greater than or equal to 1,600 nt in length. We have provided the mean, median, and standard deviation of the exon lengths in a text box.

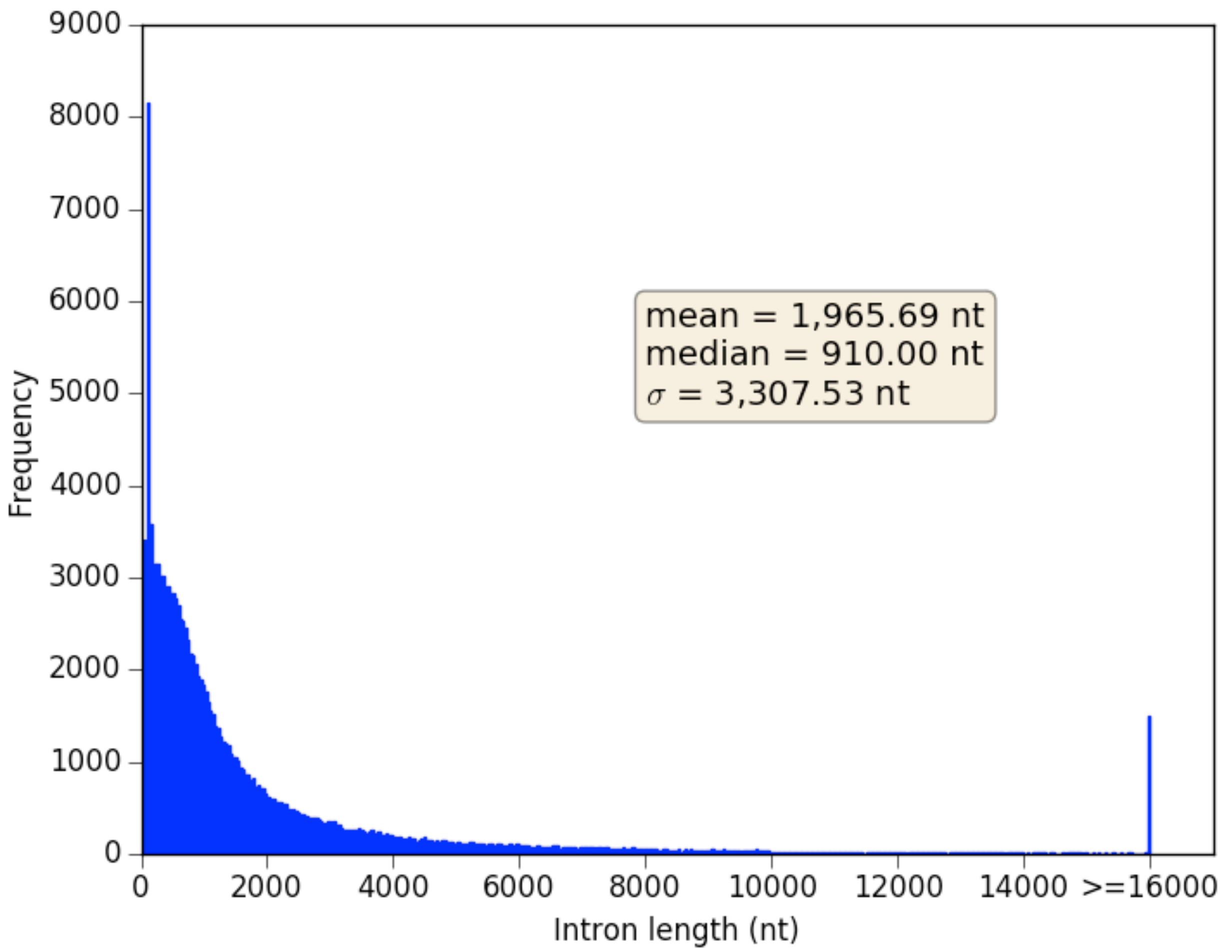

Figure S5. Histogram of the lengths of introns in genes annotated by MAKER. This is a histogram of the lengths of introns in genes annotated by MAKER. We included the introns from all of the gene annotations in the MAKER final output. We grouped the values into 400 frequency bins, one of these including all introns greater than or equal to 16,000 nt in length. We have provided the mean, median, and standard deviation of the intron lengths in a text box.

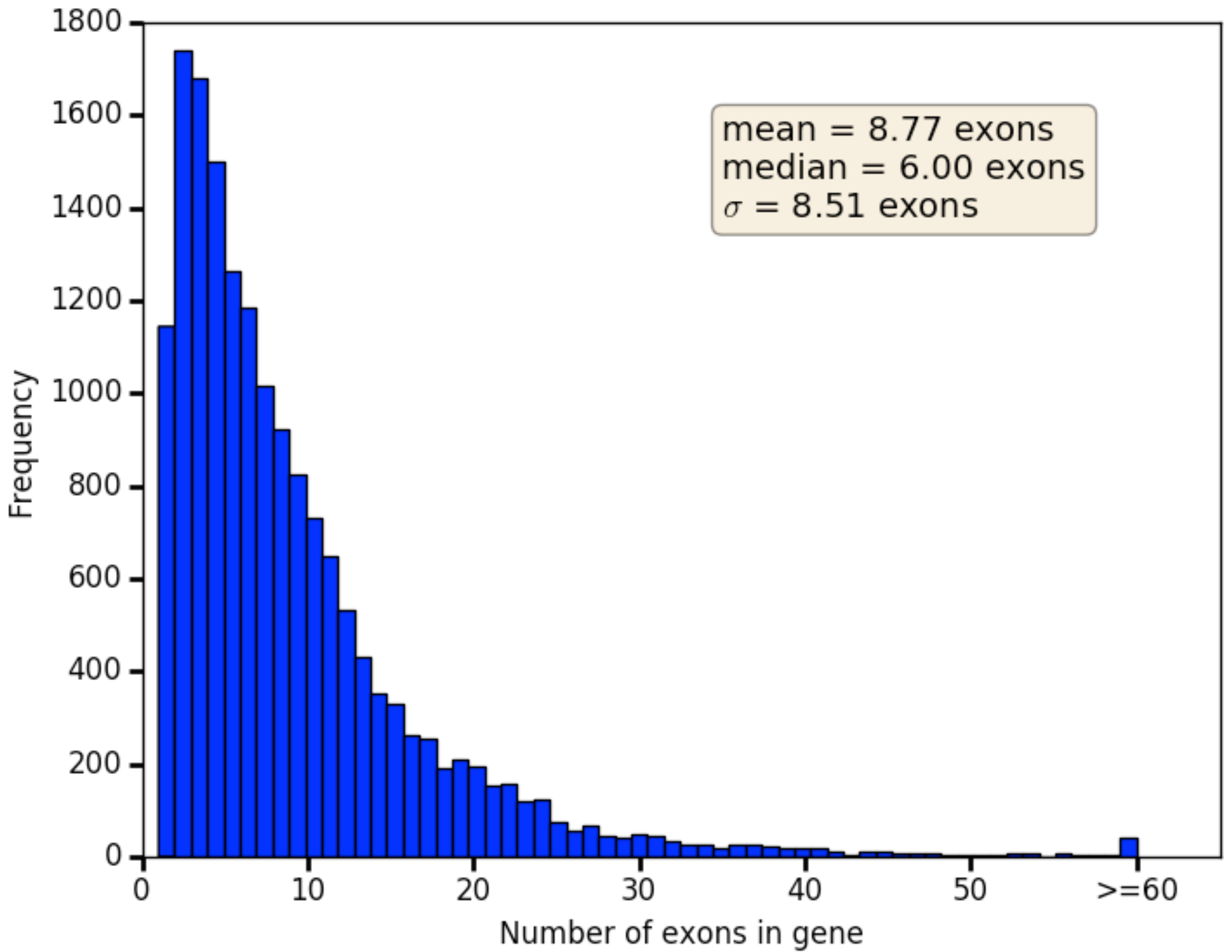

Figure S6. Histogram of the number of exons in genes annotated by MAKER. This is a histogram of the number of exons in genes annotated by MAKER. We included the exons from all of the gene annotations in the MAKER final output. We grouped the values into 60 frequency bins, one of these including all genes with greater than or equal to 60 exons. We have provided the mean, median, and standard deviation of the number of exons per gene in a text box.

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