RESEARCH ARTICLE

The transcriptome of the veiled chameleon (*Chamaeleo calyptratus*): A resource for studying the evolution and development of vertebrates

Brendan J. Pinto[1] | Daren C. Card[2] | Todd A. Castoe[2] | Raul E. Diaz Jr[3,4] | Stuart V. Nielsen[1] | Paul A. Trainor[5,6] | Tony Gamble[1,7,8]

[1]Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin

[2]Department of Biology, The University of Texas at Arlington, Arlington, Texas

[3]Department of Biological Sciences, Southeastern Louisiana University, Hammond, Louisiana

[4]Natural History Museum of Los Angeles County, Los Angeles, California

[5]Department of Anatomy & Cell Biology, Stowers Institute for Medical Research, Kansas City, Missouri

[6]Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, Kansas

[7]Milwaukee Public Museum, Milwaukee, Wisconsin

[8]Bell Museum of Natural History, University of Minnesota, St Paul, Minnesota

Correspondence
Brendan J. Pinto, Department of Biological Sciences, Marquette University, Milwaukee, WI 53233.
Email: brendan.pinto@marquette.edu

Present address
Daren C. Card, Department of Organismic and Evolutionary Biology, Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts.
and
Stuart V. Nielsen, Florida Museum of Natural History, University of Florida, Gainesville, Florida.

Abstract

Purpose: The veiled chameleon (*Chamaeleo calyptratus*) is an emerging model system for studying functional morphology and evolutionary developmental biology (evo-devo). Chameleons possess body plans that are highly adapted to an arboreal life style, featuring laterally compressed bodies, split hands/ft for grasping, a projectile tongue, turreted independently moving eyes, and a prehensile tail. Despite being one of the most phenotypically divergent clades of tetrapods, genomic resources for chameleons are severely lacking.

Methods: To address this lack of resources, we used RNAseq to generate 288 million raw Illumina sequence reads from four adult tissues (male and female eyes and gonads) and whole embryos at three distinct developmental stages. We used these data to assemble a largely complete de novo transcriptome consisting of only 82 952 transcripts. In addition, a majority of assembled transcripts (67%) were successfully annotated.

Results: We then demonstrated the utility of these data in the context of studying visual system evolution by examining the content of veiled chameleon opsin genes to show that chameleons possess all five ancestral tetrapod opsins.

Conclusion: We present this de novo, annotated, multi-tissue transcriptome assembly for the Veiled Chameleon, *Chamaeleo calyptratus*, as a resource to address a range of evolutionary and developmental questions. The associated raw reads and final annotated transcriptome assembly are freely available for use on NCBI and Figshare, respectively.

1 | INTRODUCTION

The veiled chameleon (*Chamaeleo calyptratus*) has become an increasingly important model system for studying development and evolution[1–4] As a member of the Chamaeleonidae, this species represents an intriguing and valuable example of a species with a terrestrial tetrapod body plan adapted to an arboreal ecology, highlighted by their laterally compressed bodies, zygodactyl (split) hands/ft for grasping, projectile tongue, turreted, independently moving eyes, and prehensile tail.[5] Ecologically, chameleons have undergone evolutionary shifts from inhabiting the forest floor to becoming highly adapted for an arboreal lifestyle,[6] which has entailed several major shifts in morphology and ecophysiology, including the evolution of: complex coloration and patterning,[7,8] a 4-fold variability in body size ranging from some of the smallest amniotes to the largest climbing lizards,[5] (Diaz and Trainor, 2015), diverse reproductive life histories (ranging from live birth to egg-laying, and diapause at the early gastrula stage at oviposition),[9] sexually dimorphic traits,[10] and sex determination mechanisms.[11,12]

Additionally, we have recently developed the ability to sex early-embryonic material,[11] priming further developmental studies of sexual development. Indeed, despite great potential as a model system due to being one of the most phenotypically divergent clades of tetrapods, the current lack of genome-scale resources are hindering the utility of *C. calyptratus* as a model organism in evolutionary developmental biology.[3] Thus, to help fill this gap, we sequenced, assembled, and annotated a freely available multi-tissue transcriptome resource for the veiled chameleon that includes sampling of multiple tissues, multiple sexes, and multiple developmental time points. This transcriptome resource represents the fourth transcriptome for an Acrodont reptile, families Chamaeleonidae[13] and Agamidae,[14,15] and provides a valuable resource for evolutionary developmental biology studies;[3] such as facilitating the development of RNA probes for *in situ* hybridization experiments,[16] for comparative studies of differential gene expression throughout ontogeny,[17] and for studies of gene and genome evolution.[18]

2 | RESULTS

We assessed our final transcriptome assembly using three transcriptome benchmarking methods: TransRate [v1.01][19] within DRAP; Benchmarking Universal Single-Copy Orthologs (BUSCO) [v2.0][20] with three databases using the gVolante Web service [v1.2.0];[21] and internally validated our assembly by mapping raw Illumina reads back to the final meta assembly. The TransRate assembly score is a calculated geometric mean of contig scores multiplied by the ratio of input raw reads that provide support for a given assembly.[19] This score attempts to capture the reliability of what was assembled and the completeness of the assembly. Our assembly queried a total of 70% of reference AnoCar2.0 peptides, which provided 24 921 conditional Reciprocal Best BLAST (RBB) hits, to generate a modest TransRate score of 0.1678.

Next, we used BUSCO to validate the completeness of our assembly against 3 different databases using the gVolante webservice:[21] tetrapoda, vertebrata, and core vertebrate genes (CVG). Indeed, our assembly, when compared against a database of conserved single-copy orthologs from tetrapods (3950 genes) and vertebrates (2586 genes), achieved a BUSCO score of 92.6% and 95.94%, respectively. Furthermore, when compared against the CVG database (233 genes), our assembly possesses 99.14% complete copies of this gene set (ie, missing 2 genes). When comparing this latter score with other de novo squamate transcriptomes analyzed by means of gVolante, it is only exceeded by one the Madagascar ground gecko (*Paroedura picta*), which contained 100% of CVG dataset.[22] Notably, our assembly significantly outperforms the previously published *Chamaeleo chamaeleon* transcriptome,[13] which achieved a modest score of 42.92%. In addition to its completeness, we internally validated our final assembly by mapping reads back to our transcriptome using bwa [v0.7.17][23] and calculated mapping statistics using bamtools [v2.5.1]. We successfully mapped 91.91% of our raw reads back to the final transcriptome assembly. This percent of mapped reads exceeds the average for a Trinity-only de novo assembly of 87%,[24] indicating that this transcriptome is well-assembled and is representative of the total input data used.

2.1 | Transcriptome utility

To illustrate the utility of our transcriptome assemblies, we queried the assembly for a small number of transcripts expected a priori to be present in the sequenced tissues. For example, the combined transcriptome included mRNA from adult chameleon eyes, and we, therefore, expected visual opsins to be present in the assembly. The ancestral amniote opsin complement consisted of five opsin genes, expressed in one of two cell types: vertebrate rhodopsin (RH1) in rod cells; long wavelength-sensitive opsin (LWS), short-wave sensitive 1 (SWS1), short-wave sensitive 1 (SWS2), and RH1-like 2 (RH2) in cone cells.[25] Several amniote lineages, however, have deviated from this ancestral complement and have lost one of more of their visual opsins.[25–31] Many chameleon species are both brightly colored and sexually dimorphic; thus, color vision presumably plays an important role in natural and sexual selection.[7,8]

We created a BLAST database of the assembled transcriptome in Geneious [v11.0.3][32] and queried the database with the five visual opsins from the *Anolis* genome.[33] We found a match with low E-values for each query. We created a phylogenetic dataset of visual opsin coding regions that included the *C. calyptratus* opsins, opsins from 17 other amniote species, and *Xenopus*. We used pineal opsins (OPNP) from five amniote species as an outgroup. Sequences were aligned using MUSCLE [v3.8.425][32,34] and we reconstructed a maximum-likelihood phylogeny using RAxML-HPC BlackBox [v8.2.9][35] implemented on the CIPRES Science Gateway.[36] Nodal support was estimated using rapid bootstrapping with RAxML's automatic bootstopping function, which stopped after 150 pseudo-replicates.[37]

Similar to birds and non-gecko lizards (eg, *Anolis*, *Pogona*, *Shinisaurus*, and *Ophisaurus*), we discovered that *C. calyptratus* possesses all five ancestral opsins that were present in the most recent common ancestor of tetrapods (Figure 1). Phylogenetic relationships among the five visual opsin gene families were consistent with other recently published trees;[27,30,38] for each of the five opsins, *C. calyptratus* sequences formed a clade with orthologous *Pogona* sequences, which reflects the close phylogenetic affinity of agamids and chameleons as sister taxa.[39,40] Of interest, in SWS1 we also identified the presence of a phenylalanine at residue 86 (*sensu*)[41] that is indicative of UV sensitivity in *C. calyptratus*, which is consistent with the presence of a UV sensitive pigment described in this species.[42]

3 | DISCUSSION

We present an annotated, multi-tissue transcriptome for the Veiled Chameleon, *Chamaeleo calyptratus.* Our analyses suggest that this resource provides a valuable and reasonably
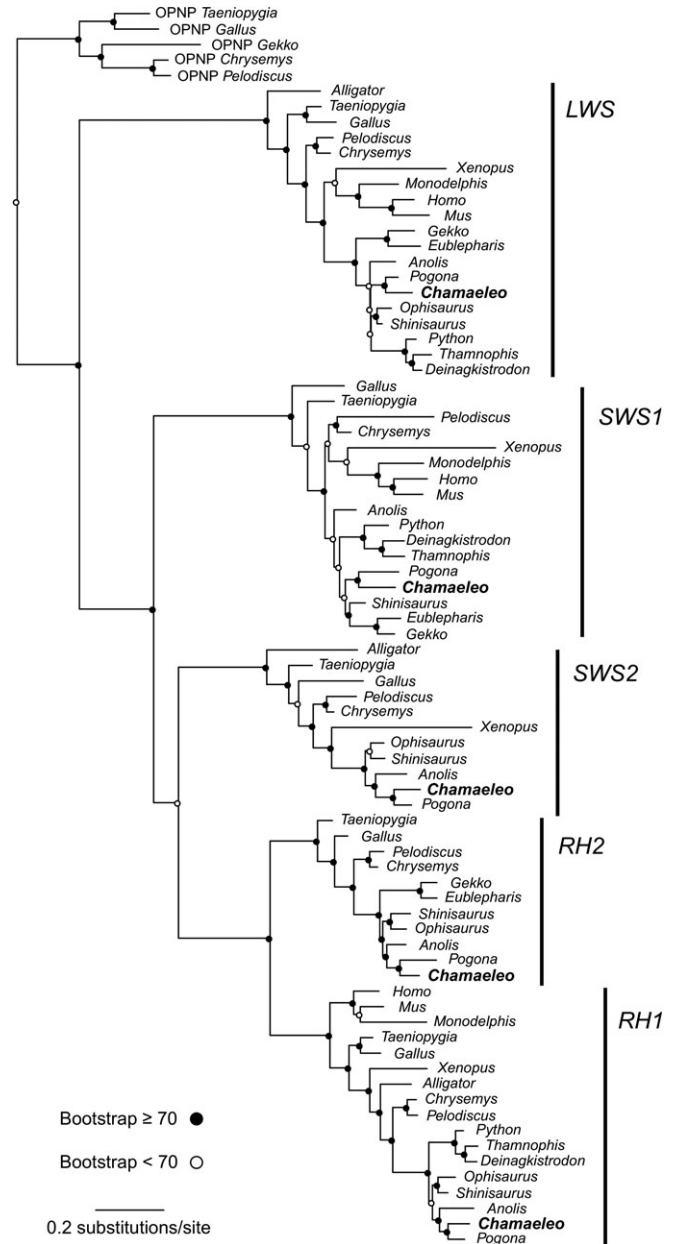

FIGURE 1   A maximum-likelihood phylogenetic reconstruction of the visual opsins of *C. calyptratus* and other tetrapods, including: vertebrate rhodopsin (RH1), long wavelength-sensitive opsin (LWS), short-wave sensitive 1 (SWS1), short-wave sensitive 2 (SWS2), and RH1-like 2 (RH2)

comprehensive catalog of transcripts for this species, as well as for comparative analyses with other vertebrates. Indeed, this transcriptome assembly contains over 90% of the benchmarking genes in three different gene ortholog databases and all five opsin genes present in the ancestor to all tetrapods. Furthermore, the availability of these data provides new important resources to address a range of evolutionary and developmental questions. For example, squamate reptiles remain the largest clade (~10 000 species) in which neural crest cell development has not been studied to any considerable

degree.[3] Neural crest cells comprise a migratory progenitor cell population and are considered a conduit through which evolution drives variation and morphological innovation.[43,44] Chameleons represent one of the most phenotypically divergent clades of tetrapods, and this transcriptome contains annotated transcripts of standard neural crest cell markers including, *tfap2*, *foxd3*, *snai1*, *snai2*, *sox9*, *sox10*, *zeb2*.

In the future, this resource should, therefore, provide important insights into body plan evolution for a taxon with a modified cranial skeleton and complex skin pigmentation. Thus, this transcriptome will be a valuable resource to the scientific community by facilitating the development of RNA probes and their use in comparative studies of differential gene expression throughout ontogeny, for comparative studies of gene and genome evolution, in the annotation and editing of genome(s), and analyses of gene function.

4 | EXPERIMENTAL PROCEDURES

4.1 | Samples

We extracted RNA from seven distinct tissues from seven different *C. calyptratus* individuals, and prepared RNAseq libraries using two preparation methods. First, we extracted RNA from three whole embryos preserved in RNA later using the Qiagen RNeasy Mini Kit and manufacturer's protocol. The embryonic ages corresponded to phenotypic landmarks: (a) Gastrula (embryonic day ~65), (b) early somite stage (~15 somites; ~77 embryonic days), and (c) early limb bud stage (~84 days of development) incubated at 26-28°C.[5] RNA was pooled from all three stages into a single RNAseq library for sequencing. Embryo RNAseq library preparation was outsourced to SeqWright [now NeoGenomics] (Houston, TX). These libraries were constructed using a non-stranded, poly-A RNAseq library protocol with TruSeq universal adapters. We also extracted RNA from four adult tissues: one male eye and testis, and one female eye and ovary, all stored in TRIzol and frozen at −80°C immediately after removal. We followed a modified version of an RNA extraction protocol for extracting RNA from TRIzol preserved tissue.[45]

Briefly, TRIzol preserved tissue was homogenized with a plastic disposable pestle over a ~7-min period at room temperature to allow for complete dissociation of nucleoprotein complexes. Then, we added chloroform and centrifuged at 4°C, mixed the aqueous phase with equal parts 70% EtOH, and transferred to a Qiagen RNeasy Mini kit for purification. We prepared RNAseq libraries using the KAPA Stranded mRNA-Seq Kit for Illumina Platforms (KR0960 [v5.17]) using oligo-dT beads for mRNA enrichment. These four libraries were prepared and indexed separately.

4.2 | Sequencing

The embryo and adult tissue libraries were sequenced on an Illumina HiSeq 2500 at SeqWright (Houston, TX) (paired-end 100 bp reads) and at the Medical College of Wisconsin (Milwaukee, WI) (paired-end 125 bp reads), respectively. Total Illumina data included 287 739 976 paired sequencing reads (the number of reads for each tissue is listed in Table 1). Quality statistics and scores from raw data were calculated using FastQC software.[46]

4.3 | Transcriptome assembly

We assembled a de novo transcriptome using the De novo RNA-Seq Assembly Pipeline (DRAP) [v1.91],[47] which is a compilation of assembly and quality control scripts using several software packages. Briefly, DRAP uses Trinity [v2.4.0][48] to trim, normalize, and assemble raw Illumina reads into a de novo transcriptome. This Trinity assembly is then edited, filtered, mapped, compacted, and quality assessed using a series of tools within DRAP: seqclean [v2011.02.22],[49] cd-hit [v4.6],[50] TGICL [v2.1],[51] TransDecoder [v2.0.1],[52] bwa [v0.7.15],[23] eXpress [v1.5.1],[53] BlatSuite [v34.0],[54] and Exonerate [v2.2.0].[55] Overall, DRAP uses these tools to generate an assembled transcriptome with less redundancy, without compromising the completeness or quality of the assembly. Reference peptide sequences provided for reference mapping in all assemblies and assessment reports were from the Green Anole (*Anolis carolinensis*)[33] downloaded from Ensembl (AnoCar2.0). We assembled transcripts from the

TABLE 1 Individual, sample tissue, sex, raw-read pair data, and accompanied NCBI SRA accession numbers for the raw sequence data used in this study

Individual	Tissue	Sex	Read length	Number of raw-read pairs	Accession numbers
TG2597	Eye	M	126	43 558 381	SAMN08358867
TG2785	Testis	M	126	25 988 093	SAMN08358868
TG2872	Eye	F	126	30 562 533	SAMN08358869
TG2786	Ovary	F	126	16 701 737	SAMN08358870
–	Embryos	–	100	170 929 232	SAMN08358871
Total				287 739 976	PRJNA429753

TABLE 2   Read QC before transcriptome assembly for each independent assembly (four adult tissues and three embryo stages) and the final meta-assembly

Dataset	Low-quality reads	Trimmed length range	Normalized read pairs	Assembled contigs
Tissues	0	32-126	22 363 082	242 734
Embryos	0	32-100	30 230 293	76 220
Meta	0	32–126	52 593 375	82 952

embryos and adult tissues, separately (Table 2). Then, we merged these two assemblies and filtered redundant transcripts using the runMeta function in DRAP. We used the runAssessment function in DRAP to generate quality scores and assembly statistics on all three assemblies. Our final combined transcriptome contained 82 952 transcripts with a total length of 124 660 559 base-pairs (bp), with transcripts ranging from 201 bp to 27 699 bp in length (Table 3).

TABLE 3   De novo transcriptome assembly statistics for the annotated *C. calyptratus* constructed using DRAP (Cabau et al., 2017)

Assembly statistic	Value
Total number of paired reads[a]	37 949 005
Number of assembled contigs	82 952
GC content	0.45%
Contig N10	5675
Contig N50	2276
Contig N90	690
Contig L50	16 508
Median contig length	1030
Mean contig length	1502.8
Number of contigs with ORF	29 506

Statistic abbreviations:
N″X": shortest contig length at "X"% of the total assembly; L50: smallest number of contigs whose length sum produces N50; ORF: open reading frame.
[a]To assess quality of final transcriptome, merged reads from Table 2 were concatenated and normalized again to reduce redundancy, leading to the discrepancy between this number and the number of normalized read pairs from Table 2.

TABLE 4   Annotation summary for the *C. calyptratus* transcriptome presented in this study (transcripts can be annotated by means of multiple databases)

Annotation of the DRAP transcriptome assembly	
Annotated genes	**55 346**
Transcripts with SwissProt annotation	39 878
Transcripts with predicted GO term annotation	47 037
Transcripts with RBB *Anolis* annotation	13 359
Transcripts with One-way *Anolis* annotation	15 455
Unannotated transcripts	27 606

4.4 | Assembly annotation

We used TransDecoder [v4.0.0][56] to identify candidate open reading frames (ORFs; coding-regions) within the de novo transcripts we assembled. We used several homology-based searches to annotate these proteins with gene identities, which were stored in a Trinotate SQLite database [v3.0.2]:[57] (1) HMMer[58] search against pfam database [v31.0],[59] (2) BLASTp and BLASTx searches against the SwissProt database (31 Jan 2018 release), and (3) both Reciprocal Best BLAST (RBB; e-value threshold of 1e-3) and one-way BLASTp (ONEWAY; e-value threshold of 1e-5) searches against protein models for AnoCar2.0. The annotation report is provided in Table 4. FASTA formatted data file headers were edited before and after Trinotate annotation to produce our final transcriptome file using SeqKit software package [v0.7.2].[60]

4.5 | Data availability

Sequence data are available in the NCBI SRA (Table 1) and associated with BioProject PRJNA429753. All three transcriptome assemblies (embryo, tissue, and combined assembly) are available in the Figshare repository associated with this article; as is the SQLite database associated with the transcriptome annotations Pinto BJ, doi:10.6084/m9.figshare.7327067.v2.

5 | CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

analyzed transcriptomes, and drafted the manuscript. D.C.C. annotated the final transcriptome. S.V.N. and R.E.D. extracted RNA from embryos and adult tissues, respectively, and S.V.N constructed adult tissue libraries. T.G. aligned and generated opsin gene tree. T.A.C., R.E.D., P.A.T., and T.G. conceived and designed experiments and project goals. All authors contributed to and approved the final manuscript.

ORCID

Brendan J. Pinto https://orcid.org/0000-0002-4243-5788
Raul E. Diaz Jr https://orcid.org/0000-0001-9107-124X
Paul A. Trainor https://orcid.org/0000-0003-2774-3624
Tony Gamble https://orcid.org/0000-0002-0204-8003

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