Research Article

Leveraging Comparative Genomics to Identify and Functionally Characterize Genes Associated with Sperm Phenotypes in *Python bivittatus* (Burmese Python)

Kristopher J. L. Irizarry¹ and Josep Rutllant²

¹The Applied Genomics Center, Graduate College of Biomedical Sciences, College of Veterinary Medicine, Western University of Health Sciences, 309 East Second Street, Pomona, CA 91766, USA
²Molecular Reproduction Laboratory, College of Veterinary Medicine, Western University of Health Sciences, 309 East Second Street, Pomona, CA 91766, USA

Correspondence should be addressed to Kristopher J. L. Irizarry; kirizarry@westernu.edu

Received 30 September 2015; Revised 30 January 2016; Accepted 18 February 2016

Academic Editor: Jerzy Kulski

Copyright © 2016 K. J. L. Irizarry and J. Rutllant. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Comparative genomics approaches provide a means of leveraging functional genomics information from a highly annotated model organism’s genome (such as the mouse genome) in order to make physiological inferences about the role of genes and proteins in a less characterized organism’s genome (such as the Burmese python). We employed a comparative genomics approach to produce the functional annotation of *Python bivittatus* genes encoding proteins associated with sperm phenotypes. We identify 129 gene-phenotype relationships in the python which are implicated in 10 specific sperm phenotypes. Results obtained through our systematic analysis identified subsets of python genes exhibiting associations with gene ontology annotation terms. Functional annotation data was represented in a semantic scatter plot. Together, these newly annotated *Python bivittatus* genome resources provide a high resolution framework from which the biology relating to reptile spermatogenesis, fertility, and reproduction can be further investigated. Applications of our research include (1) production of genetic diagnostics for assessing fertility in domestic and wild reptiles; (2) enhanced assisted reproduction technology for endangered and captive reptiles; and (3) novel molecular targets for biotechnology-based approaches aimed at reducing fertility and reproduction of invasive reptiles. Additional enhancements to reptile genomic resources will further enhance their value.

1. Introduction

Reptiles represent a diverse and biologically distinct group of vertebrates for which most species have yet to be systematically studied. Over the last few decades, reptiles in general, and snakes in particular, have grown in popularity among owners and breeders. It is worth noting that reptiles are of ecological interest as both endangered and invasive species. For example, in 2015, California Department of Fish and Wildlife listed ten distinct reptile species as either endangered or threatened, including four species of snakes: *Charina bottae* (southern rubber boa), *Thamnophis gigas* (giant garter snake), *Thamnophis sirtalis tetrataenia* (San Francisco garter snake), and *Masticophis lateralis euryxanthus* (alameda whip-snake). At the same time, California Department of Fish and Wildlife classifies other reptiles as invasive species, such as *Nerodia fasciata* (southern watersnake). The identification of genetic and genomic resources for use in reptiles can accelerate research and ultimately enhance knowledge of their unique biology. Deciphering reptile reproductive biology can provide avenues for facilitating successful breeding in endangered species and, at the same time, may offer insights into reducing the reproduction of invasive species.

Two species of the genus *Python* have recently become the focus of genomics level investigations. Castoe et al. sequenced the genome of the Burmese python as a reptilian model organism with the expectation that the sequence data would provide insight into unique aspects of reptilian physiology and evolution [1]. Similarly, whole transcriptome studies in ball python have led to the production of genomics resources...
which can be used to further study this species [2]. Recent investigations into python physiology have identified rapid gene expression changes associated with basic physiological processes such as eating [3]. For example, the Burmese python exhibits unique organ and metabolic adaptations which have been characterized recently at the molecular and genetic levels [4].

Python regius (ball python or royal python) is a relatively small member of the Python family which has become an extremely popular pet over the last 15 to 20 years due to the tremendous expansion of color variations that have been produced. P. regius is a relatively small species in length (rarely over 2 meters) and adapts easily to being raised in captivity. The name “ball python” is derived from the fact that this species curls up in a ball whenever it is approached or handled, making it particularly easy to manage in captivity.

Python molurus bivittatus (Burmese python) has been a popular pet in the United States since the 1990s due to their attractive color patterns, docile nature, and large size [5]. Size-wise, the Burmese python is one of the largest snakes in the world and can reach over 23 feet in length and weights of over 200 pounds. Some of these giant snakes have been illegally released by their owners into the wild due to the difficulty to handle them and the lack of alternate housing or sheltering. Nowadays, although native to Southeast Asia, these snakes are exotic (nonnative) species in areas like South Florida (e.g., Everglades National Park) and they are also considered invasive species [6] since they are not constrained by natural factors. Consequently, due to their potential to harm invaded environments (wildlife and ecosystem), efforts are underway to reduce their numbers in these sensitive environments. Because of their large size, Burmese pythons have few predators, and subsequently their predation upon native species is decreasing the native populations to the level of being threatened or endangered [7, 8]. The impact of the invasive Burmese pythons on the normal wild life is serious concern. Dorcas et al. describe the severe reduction in mammals in the Florida Everglades due to the pythons growing population size [9]. Novel genetic strategies are being employed to monitor the Burmese python. Recently, PCR-based detection methods have been employed to detect Burmese python DNA in environmental water samples, such as marshes, streams, swamps, and lakes [10].

In contrast to invasive reptiles, endangered reptiles are exhibiting decreased numbers in their native ecosystems. Populations of endangered reptiles may suffer from inbreeding due to reduced populations or even reproduction in a captive setting such as a zoo [11]. Hussain et al. developed methods to quantify damage to semen at specific steps in the preservation process [12]. The work was carried out in mammals; however, the approach is viable for reptiles as well. Ruiz-Lopez et al. describe the relationship between homozgyosity, heterozygosity, and inbreeding depression [13]. These undesirable genetic issues arise when populations are endangered and can contribute to decreased reproductive fitness, including poor sperm function, reduced motility, and decreased sperm numbers in the endangered population. Birds have been used as models for developing reproductive technology such as artificial insemination and extenders capable of improving the longevity and value of cryopreserved semen from endangered species. For example, a 2009 study evaluated post-thaw semen quality in wild-caught Griffon vultures and determined that cryopreservation of semen is a useful tool in the conservation of endangered species genetic resources [14].

Assisted reproductive technology helped immensely to improve genetic pools in farm and domestic animals for several decades; however, although recognized as an important strategy to enhance diversity and increase captive populations of endangered animals, these techniques are rarely applied to reptiles [15] and much less to the specific field of snakes [16]. Reproduction in snakes differs from mammalian reproduction in many distinct ways, but the most significant difference is that spermatozoa can be stored in the female genital tract for months, if not years, before fertilization [17]. Studies related to the development of assisted reproductive techniques in snakes have been ignored and only few reports on semen collection [18, 19], sperm preservation [20], and artificial insemination [15] have been the focus of large research efforts.

Comparative genomics has been successfully employed in previous studies to identify physiologically important genes in one organism based on the annotation provided by a model organism genome. The comparative genomics approach was developed and heavily leveraged in the 1990s to facilitate functional annotation of large-scale human EST data produced during the effort to sequence the human genome [21]. Functional information about gene-phenotype relationships in model organisms were shown to be extremely valuable in deciphering the consequence of identified mutations in human genes [22]. Comparative genomics approaches became more widespread following the sequencing of multiple genomes and the emerging need to characterize unknown genes [23]. For example, genes sequenced from hamster testis were used to identify and characterize genes previously uncharacterized in human, mouse, rat, and pufferfish genomes [24]. Beyond simply facilitating the identification of novel genes, comparative genomics approaches have been effectively used to characterize individual proteins involved in sperm phenotypes, such as the sperm mitochondrial cysteine-rich protein, SMCP [25]. Such approaches have also been effective in identifying economically important reproductive traits in agriculturally important species, such as the pig [26]. Similarly, the characterization of 1227 genes in the domestic cat was achieved using a comparative genomics approach in which cat genes having phenotypically characterized orthologs in the mouse were annotated with developmental, clinical, and nutritional phenotypes [27].

A number of bioinformatics resources have been developed to facilitate the use of comparative and functional genomics. Ontologies, which are controlled vocabularies organized around specific parent-sibling relationships and maintained in a graph structure, provide standardized nomenclature and relationships among biologically relevant terminology for applications in bioinformatics and functional genomics [28]. One of the most widely used ontologies in genomics is the gene ontology [29]. Gene set enrichment,
Figure 1: Overview of the comparative genomics approach to identify and characterize python genes associated with sperm phenotypes. The set of mouse protein coding genes was used to select the subset of mouse genes for which phenotype annotation information was available. Starting with all mouse genes having phenotype annotation, we identified the subset corresponding to protein coding genes associated with only sperm phenotypes. This set of mouse protein sequences was subsequently used to identify the corresponding protein coding sequences in *Python bivittatus* (i.e., orthologous genes). For each protein coding sequence shared between mouse and python, a pairwise protein sequence alignment was generated and measures of sequence identity and significance were calculated. Gene ontology (GO) annotation provides gene level information about biological processes, cellular locations, and molecular functions of gene products. The existing GO annotation for each mouse gene was "added" to each python orthologous gene. Then set of python genes was analyzed for statistically significant enrichment of genes associated with particular GO annotation terms across the three GO categories (biological process, cellular compartment, and molecular function). This resulting set of annotated python genes provides additional biological, physiological, cellular, and molecular information about the roles of these genes in sperm production and function. Moreover, the annotation also offers an independent set of annotation information to help validate the python genes as truly being associated with sperm biology. Additionally, cellular pathways which are associated with the sperm associated gene set were identified along with human disorders caused by human orthologs of these genes.

such as the identification of shared biological processes among a set of differentially regulated genes in a gene expression experiment, represents one of the most widely used applications of gene ontology [30]. Gene set enrichment has been previously applied to the identification and analysis of genes associated with phenotypes [14, 31]. Other ontologies have been developed that are useful for functional annotation of genes, including the mammalian phenotype ontology [32], the human phenotype ontology [33], and the mouse-human anatomy ontology [34].

In addition to ontological resources, pathway mapping resources provide additional means of functionally annotating genes based on biologically relevant information. One of the most widely utilized pathway resources for gene annotation is the KEGG pathway database [35] which provides knowledge-based representations of biochemical pathways and protein interaction networks. Tools have been developed that facilitate the identification of KEGG pathway members from a set of genes [36, 37]. One of the most widely used tools, based on more than 21,000 citations, is the Database for Annotation, Visualization, and Integrated Discovery [38] that provides an interface for functional gene annotation including gene ontology and KEGG pathway analysis.

The relatively recent production of reptile genomic resources has opened the door to leveraging genome-scale information to address the challenges associated with endangered and invasive reptiles. In an attempt to expand the set of genetic resources available for investigating reptile biology, we have chosen to utilize a comparative genomics strategy to identify python genes likely to play a significant role in sperm development and function (Figure 1). We describe the selection of a set of mouse genes that have been previously demonstrated to modulate sperm phenotypes and use that mouse set to identify and functionally characterize a corresponding set of python genes. Although reptiles and mammals share many aspects of biology with each other, they each have also evolved unique adaptations that are found only in their respective lineages. By leveraging mammalian reproductive genetics to initiate the construction of reptile reproductive genomics tools, the long and productive history of mouse biology and genetics can be brought to bear on the emerging field of reptile reproductive genetics.
2. Materials and Methods

2.1. Comparative Genomics Approach to Identify Python Genes Associated with Sperm Phenotypes. The comparative genomics approach (Figure 1) leveraged the set of mouse genes for which phenotype information was available. Mouse genes annotated with sperm specific phenotypes were identified and python orthologs were identified as described below. Gene ontology enrichment was performed to identify gene ontology biological process, cell component, and molecular function terms associated with each gene set identified by a sperm associated phenotype. The resulting set of python genes mapped to sperm phenotypes is available as a supplemental data file, as is a second supplemental file containing the mapping of the mouse genes to the corresponding python orthologs, all of which are mapped to sperm phenotypes.

2.2. Identification of P. regius and P. bivittatus Sequences from NCBI. Publicly available DNA, mRNA, and protein Python bivittatus sequences were downloaded from the NCBI nucleotide and protein sequence databases (http://www.ncbi.nlm.nih.gov/) by searching NCBI for “Python bivittatus.” A total of 25,944 protein sequences were identified. P. regius sequences were also downloaded from NCBI using a similar query for which “Python regius” was used as search term. Although considerable sequence data exists for P. bivittatus (20,392 genes, 25,944 protein sequences, and 105,311 nucleotide sequences), relatively few sequence resources are available for P. regius (21 genes, 141 protein sequences, and 123 nucleotide sequences). Interestingly, some of the identified P. regius sequences in the database correspond to viral sequences, such as the ball python nidovirus (8 genes, 3 nucleotide sequences). Even so, we identified a set of P. regius sequences for which orthologous P. bivittatus sequences were available. Because we are specifically interested in python molecular reproductive genetics, we selected (from among the set of 21 genes) those for which published papers had previously implicated the gene as (1) involved in sperm related biology or (2) being associated with variation in sperm parameters, or (3) being associated with variation in fertility in another species (Table 1).

2.3. Selection of Mouse Genes Associated with Sperm Phenotypes. Mouse gene and protein identifiers along with protein sequences were downloaded from the Ensembl database (http://www.ensembl.org/). Data was loaded into a table in a relational database (MySQL) containing phenotype annotation from the Mammalian Phenotype Browser (http://www.informatics.jax.org/searches/MP_form.shtml). Database queries in SQL provided a mechanism for selecting all mouse gene identifiers linked to specific phenotypes. The output from the database contained individual gene-phenotype relationships. Since a single gene could be associated with more than one phenotype, the results contained unique gene-phenotype relationships even though the same genes were listed under multiple phenotypes. The list of 129 sperm related phenotypes mapped to each mouse gene (listed as mouse ensemble gene identifier) is contained in Supplemental File 1 (see Supplemental File 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/7505268).

2.4. Mapping Mouse Genes Associated with Sperm Phenotypes to P. bivittatus Protein Identifiers. Each mouse gene ID was used to query the database to retrieve the gene description field which contains a variety of header information including the complete title of the gene. The mouse gene title information was used to manually search through NCBI's protein database (http://www.ncbi.nlm.nih.gov/protein?term=Python%20bivittatus) for orthologous python protein sequences. Because the goal of the project was to insure the collection of genes having sperm phenotypes, any genes which were not the obvious ortholog of the mouse gene were excluded from the final dataset. For example, in some cases, a specific family member of a multigene family was sought, but the exact family member could not be found even though a number of paralogous P. bivittatus genes from the same family were located.

Finally, the blastall program was used to perform blastp analysis of the protein sequences associated with sperm phenotypes. For each orthologous mouse-pyhton gene pair, blastp calculated the bit score, generated an alignment, and calculated the percent identity. A tab-delimited text file containing each sperm associated phenotype, the Ensembl mouse gene identifier, official gene symbol, the Burmese python NCBI gene identifier, the Burmese python NCBI refid, and the description of each gene is contained in Supplemental File 1.

2.5. Pairwise Alignments, Multiple Sequence Alignments, and Phylogenetic Trees. Epididymal protein E1 sequence was obtained for the following species: Gallus gallus (chicken), Alligator mississippiensis (alligator), Chrysomys picta belli (painted turtle), Pantherophis guttatus (corn snake), Echis coloratus (palestine saw-scaled viper), Python bivittatus (burmese python), Python regius (ball python), Anolis carolinensis (Carolina anole), Equus caballus (domestic horse), Canis familiaris, Sus scrofa, Homo sapiens, Pan troglodytes, Felis catus, Mus musculus (mouse), Bos taurus (cow), Oncorhynchus mykiss (trout), Bombus impatiens (eastern bumble bee), Thelohanelus kitauei (fish parasite). The mitochondrial cytochrome b protein sequence was obtained for multiple sequence alignment across 17 species (Figure 2(c)). The equine sequence was taken from Equis przewalskii, the feral horse, instead of the domestic horse. Alignments were generated using CLC Sequence Viewer 6 (http://www.clcbio.com/products/clc-sequence-viewer/). Phylogenetic trees were produced using the tree construction algorithm in CLC Viewer 6.
nonconserved regions are shown in red. Sequences from the snake species Figure 2: (a) Multiple sequence alignment of epididymal protein E1. Twenty species representing fish, parasite, insect, snake, turtle, lizard, alligator, chicken, mouse, cow, horse, dog, cat, chimpanzee, and human conserved regions of the alignment are indicated in blue while nonconserved regions are shown in red. Sequences from the snake species Ophiothrix aenetus, Pantherophis guttatus, Echis coloratus, Python regius, and Python bivittatus all contain a 5-amino-acid insertion (KRGEM in pyton species) within the first ten amino acids of the alignment in comparison to other reptiles and mammalian species. Similarly, the primate lineage, represented by Homo sapiens and Pan troglodytes, exhibits a 2-amino-acid insertion (HL) at the very end of the alignment. Thelohanellus kitauei (an aquatic invertebrate parasite) and Bombus impatiens (bee) provide evidence for an ancient role of this gene in the common ancestor of insects, parasites, and vertebrates. (b) Phylogenetic tree of epididymal protein E1. The multiple sequence alignment shown in Figure 3(a) was used to generate a phylogenetic tree using a bootstrapping approach. The number near each root or interior node of the tree indicates how many times the same subtree, as shown in the image, was obtained when the input sequences were sampled during the bootstrapping process. In this tree, Python regius and Python bivittatus are grouped together in all 10,000 trees produced during the phylogenetic tree construction process. Similarly, the Homo sapiens and Pan troglodytes subtribe also exhibits a count of 10,000. The scale bar below the tree provides an estimate for sequence evolution rate between the taxa. (c) Multiple sequence alignment of mitochondrial cytochrome b protein. The cytochrome b protein was aligned across multiple species to visualize the sequence relationship between Python regius and Python bivittatus in the context of other species. Conserved regions of the alignment are indicated in blue while less conserved regions are shown in red. The species representing snakes exhibit an absence of the 5 amino acids within the first ten amino acids of the alignment. This sequence feature is not observed in other reptiles, Gallus gallus (chicken) and Oncorhynchus mykiss (trout), or in mammals. Thus, there is the possibility that certain aspects of biology and/or physiology may be uniquely shared among snakes.
Figure 3: (a) Number of genes in each phenotype. The number of genes in each phenotype is shown in histogram. Within each phenotype, no gene is duplicated. However, a gene may appear in more than one phenotype. The sum of the counts is 129 corresponding to 129 gene-phenotype relationships. The number of distinct genes is 98. (b) Average percent identity for the set of genes within each phenotype. The average percent identity was calculated for each phenotype. The percent identity for each *P. bivittatus* protein sequence was determined by using BLASTP to align each *P. bivittatus* protein sequence against its corresponding mouse protein ortholog. Only the single top scoring blast hit was used to determine identity. Average identity was calculated for each phenotype by summing the individual identities within each phenotype and dividing by the total number of genes within each phenotype. (c) Standard deviation of the “average percent identity” in each phenotype. The population standard deviation of percent identity was calculated for each phenotype. The standard deviations range from a low of 7.7 to a high of 20.46. The two phenotypes with the lowest standard deviations are “impaired sperm capacitation” and “absent sperm flagella.”
Table 1: Python orthologs of genes previously associated with sperm function and/or traits along with literature references.

| Gene name                       | *P. regius* (Genbank IDs) | *P. bivittatus* (Genbank IDs) | Reference |
|---------------------------------|---------------------------|-------------------------------|-----------|
| Epididymal secretory protein type E1 | gil|698375281 gb|JAC94921.1 | 99% 8e-114 | gil|602671876 ref|XP|007441448.1 | [39] |
| Complement C3                   | gil|698375324 gb|JAC94937.1 | 95% 0.0 | gil|602634163 ref|XP|007423955.1 | [40] |
| Phospholipase B                 | gil|698375293 gb|JAC94925.1 | 96% 0.0 | gil|602641944 ref|XP|007427768.1 | [41] |
| L-Amino acid oxidase            | gil|698375301 gb|JAC94928.1 | N/A N/A | N/A N/A | [42] |
| Cysteine-rich secretory protein A | gil|698375279 gb|JAC94920.1 | 95% 1e-166 | gil|602662716 ref|XP|007436999.1 | [43] |
| Kallikrein                      | gil|698375306 gb|JAC94930.1 | 97% 0.0 | gil|602667543 ref|XP|007439332.1 | [44] |
| Factor X                        | gil|669634856 gb|JAC89993.1 | 94% 0.0 | gil|602632281 ref|XP|007423033.1 | [45] |
| Alpha enolase                   | gil|5305427 gb|AAD41646.1 | 88% 0.0 | gil|60265461 ref|XP|007432114.1 | [46] |
| Cystatin F                      | gil|698375284 gb|JAC94922.1 | 97% 7e-115 | gil|602642301 ref|XP|007427941.1 | [47] |
| Cathepsin D                     | gil|698375257 gb|JAC94912.1 | 98% 0.0 | gil|602659329 ref|XP|007435358.1 | [48] |
| NADH dehydrogenase subunit 4 (mitochondrion) | gil|74310582 ref|YP|313703.1 | 89% 0.0 | gil|511768956 ref|YP|008083608.1 | [49] |
| Cytochrome B (mitochondrion)    | gil|74310585 ref|YP|313706.1 | 94% 2e-63 | gil|224815091 gb|ACN65710.1 | [50] |
| ATP synthase F0 subunit 6 (mitochondrion) | gil|74310578 ref|YP|313699.1 | 90% 5e-126 | gil|511768952 ref|YP|008083604.1 | [51] |

Python orthologs of genes previously associated with sperm function and/or traits along with literature references. Among a set of 21 *P. regius* gene sequences available within the NCBI database, 13 were implicated in sperm function and/or traits based on previous publications in other species. The table contains the *P. regius* NCBI identifiers and corresponding orthologous *P. bivittatus* gene identifiers (for genes in which the ortholog was identified). For each orthologous pair, the protein percent identity and e-value obtained from the BLASTP analysis of the sequences are provided. Additionally, each *P. regius* gene identified has a reference implicating the gene in sperm related biology in another species. Together these genes represent evidence that *P. bivittatus* and *P. regius* exhibit high sequence identity among these genes. Specifically, 4 out of the 12 identified ortholog pairs have identity of 97% or greater (epididymal secretory protein E1, Kallikrein, Cystatin F, and cathepsin D), while just two ortholog pairs have percent identity below 90% (alpha enolase having 88% and mitochondrial NADH dehydrogenase subunit 4 with 89%).

2.6. Functional Gene Ontology Enrichment Analysis of Genes Associated with Sperm Phenotypes. Gene set analysis was performed on the set of 98 genes associated with phenotypes using the online bioinformatics package DAVID (https://david.nicifcr.gov/). The DAVID bioinformatics resource provides genome level functional annotation of genes and data sets through a web-based interface [52]. It is an ideal resource that facilitates the identification of biologically relevant signals in large-scale genomics data sets [53].

Mapping of mouse gene identifiers to python protein identifiers provided a means to accomplish analysis of the python genes using the DAVID mouse gene identifiers since the DAVID database relies upon established gene ontology associations with gene identifiers. At the time of writing, the DAVID database does not handle analyses using python gene or protein identifiers. Running the functional genomic analysis using the mouse gene identifiers as surrogates for the orthologous python genes enabled the gene ontology analysis to be accomplished with the DAVID software.

Gene ontology enrichment terms were identified using a *p* value < 0.05 and/or Benjamini probability < 0.05. Because the purpose of the DAVID software suite is to identify biological annotations that are enriched in gene sets from gene expression studies, it is of value to use stringent statistical measures of enrichment. However, unlike a gene expression experiment, the genes identified in this study each are selected due to their individual phenotype relating to sperm development, function, and morphology. Therefore, even in cases where the *p* value is > 0.05, the functional annotation associated with a specific gene is still the true biology underlying that gene’s involvement in reproductive biology.

2.7. Characterization of Phenotype-Specific Functional Annotation in Python Sperm Genes. Additional rounds of gene set functional analysis were employed to phenotype-specific biological annotations. Specifically, gene ontology enrichment terms were identified using a *p* value < 0.05 and/or Benjamini probability < 0.05 for each of the ten sperm related
phenotypes. The analysis was carried out using the DAVID bioinformatics software tool. Gene sets from each of the ten sperm phenotypes were analyzed and the results were compared to the functional annotation identified in the 98-gene set. As was considered in the analysis of the 98-gene set, annotation terms relating to reproduction and fertility were included in the functional annotation of genes even in cases where the \( p \) value was \( >0.05 \).

2.8. Visualization of Phenotype-Specific Functional Annotation in Python Sperm Genes. Visualization of gene ontology annotation was accomplished using Revigo (Supek 2011) which creates two-dimensional scatterplots and tree maps that organize the annotations via their semantic relationships with one another. The gene ontology biological process, cellular compartment, and molecular function terms identified via the characterization of phenotype-specific annotation were used as input for Revigo. The \( p \) values were converted to \(-\log(p \text{ value})\) and included as scores for each GO annotation. Parameters were set to reflect that \(-\log(p \text{ value})\) was higher for the score with the GO annotation was considered higher scoring if the associated \(-\log(p \text{ value})\) was higher (this is in contrast to \( p \) values in which lower values are better scores). Additionally, the analysis was performed using the GO database from Homo sapiens because it contained the greatest number of genes from the set of sperm associated phenotypes (97 out of 98) compared to Mus musculus and Gallus gallus. Since enrichment metrics utilize the hypergeometric distribution for calculating statistical significance, H. sapiens was considered more appropriate based on inclusion of sperm associated genes represented in the Revigo resource. The SimRel measure of semantic similarity was used for the analysis.

2.9. KEGG Pathway Enrichment Analysis of Genes Associated with Sperm Phenotypes. KEGG database provides a repository for genes associated with cellular and signaling pathways [54] which can be used to decipher gene functions. Pathway enrichment analysis was performed on the set of 98 genes associated with sperm phenotypes using the online bioinformatics package DAVID (https://david.ncifcrf.gov/) [55, 56].

2.10. Public Release of Data and Functional Annotation Associated with This Study. The authors of this project believe that the benefit of genomics and genetic resources is best accomplished when such resources are freely made available to the research community. Subsequently, the set of mouse and python gene identifiers, along with their orthologous mappings and functional associations with specific phenotypes, have been made freely available to the research community through the supplemental data associated with this publication (Supplemental File 1 and Supplemental File 2). Specifically these supplemental resources include tab-delimited files with gene ontology (biological process, cellular compartment, and molecular function) enrichment results for the sperm associated genes (Supplemental File 3, Supplemental File 4, and Supplemental File 5, resp.) as well as a FASTA file containing the protein sequences for Python bivittatus along with the corresponding sperm associated phenotype for each sequence (Supplemental File 6).

3. Results

3.1. Analysis of Protein Sequence Identity between Python regius and Python bivittatus. To explore the possibility that P. regius and P. bivittatus exhibit sufficient genetic similarity to justify using one species as a genetic model for the other species, we assessed the level of sequence identity among a set of protein coding sequences, mitochondrial DNA sequences, and protein sequences. We aligned the mRNA for epididymal protein E1 and assessed the extent of identity between the two species. The length of protein sequence was 153 amino acids long in both species. The aligned sequences covered the full length of each protein with an identity of 98% corresponding to 1189 identities with 6 gaps. This alignment produced a bit score of 2102 and an \( e \)-value of 0. Pairwise BLASTP was used to assess the identity for the pairwise protein alignment for epididymal protein E1 between the two python species. The length of protein sequence was 153 amino acids long in both species. The aligned sequences covered the full length of each protein with an identity of 98% corresponding to 1189 identical amino acids aligned with 0 gaps. A set of 12 proteins implicated in sperm related functions were selected for pairwise alignment between P. regius and P. bivittatus orthologs in order to assess the extent of protein sequence identity between the two species (Table 1). Together these orthologous alignments provide insight into the genetic relationship between these two python species. Four out of the twelve genes identified exhibited identity of 97% or greater (epididymal secretory protein E1, Kallikrein, Cystatin F, and cathepsin D), while just two ortholog pairs have percent identity below 90% (alpha enolase having 88% and mitochondrial NAD dehydrogenase subunit 4 with 89%).

3.2. Multispecies Sequence Alignments and Construction of Phylogenetic Trees across Taxa. To gain a better appreciation for the relationship that exists among taxa, with regard to the proteins implicated in sperm function, multiple sequence analysis was performed for two protein coding sequences: epididymal protein E1 (Figures 2(a) and 2(b)) and mitochondrial cytochrome b (Figure 2(c)). Sequences from the snake species (Ophodrys aestivus, Pantherophis guttatus, Echis coloratus, Python regius, and Python bivittatus) all contain a 5-amino-acid insertion (KRGEM in python species) within the first ten amino acids of the epididymal protein E1 alignment (Figure 2(a)) in comparison to other reptiles and mammalian species. Similarly, the primate lineage, represented by Homo sapiens and Pan troglodytes, exhibits a 2-amino-acid insertion (HL) at the C-terminal end of the alignment (Figure 2(a)). Thelohanellus kitauei (an aquatic invertebrate parasite) and Bombus impatiens (bee) provide evidence for an ancient role of this protein coding gene in the common ancestor of insects, parasites, and vertebrates. The multiple sequence alignment shown in Figure 2(a) was used to generate a phylogenetic tree using a bootstrapping approach (Figure 2(b)). The number near each root or interior node of the tree indicates how many times the same subtree, as shown in the image, was obtained
when the input sequences were sampled during the bootstrapping. Larger numbers indicate a greater percent of bootstrapped trees contained in the same tree organization as depicted in the figure. *Python regius* and *Python bivittatus* are grouped together in all 10,000 trees produced during the phylogenetic bootstrapping tree construction process. The only other species, for which 10,000 iterations of tree construction resulted in the two species being paired together 100% of the time, are *Homo sapiens* and *Pan troglodytes*. The common evolutionary relationship between the snake species exhibiting the 5-amino-acid insertion at the beginning of the alignment is also characterized by the magnitude of the number at the subnode of the tree which contains these species.

The mitochondrial cytochrome b protein sequence was obtained for multiple sequence alignment across 17 species (Figure 2(c)). The equine sequence was taken from *Equus przewalskii*, the feral horse, instead of the domestic horse. Upon inspecting the alignment, it was apparent that the snake sequences diverged from the nonsnake species. However, in the case of mitochondrial cytochrome b, the snakes (*P. bivittatus, P. regius, P. guttatus*, and *E. omanensis*) exhibit two short deletions within the first 30 amino acids of the alignment, in contrast to the insertion identified in epididymal protein E1. Although sequence divergence within these regions of the alignment is evident among the other species, neither the nonsnake reptiles nor the mammals exhibit the gapped alignment pattern observed in the snakes.

3.3. Identification of *P. bivittatus* Protein Sequences Associated with Sperm Phenotypes. Through the comparative genomics approach employed, 129 gene-phenotype relationships were identified in *P. bivittatus* genes (Supplemental File 1). Initially we sought to identify 152 gene-phenotype relationships based on the phenotype annotation in the mouse. However, while attempting to identify orthologous genes in the python, 13 orthologs could not be adequately identified due to ambiguity in resolving whether some python genes were truly the orthologs, or whether what was identified in the database was a paralogous sequence.

In some cases, the identified python gene contained the annotation term “partial” in the fasta header line in NCBI databases. These sequences were still included in the final gene set (even though the sequence may not be complete). Python genes lacking the term “partial” were considered to be full length; however during our analysis it became apparent that some genes lacking the annotation “partial” did not represent full length sequences.

The final set of *P. bivittatus* gene sequences associated with sperm phenotypes included 98 distinct genes (Supplemental File 2) mapping to ten classes of phenotype (Supplemental File 6). In order to carefully maintain the relationship between phenotype and gene, our approach treated each gene-phenotype relationship as a unique data point. Subsequently the 129 gene-phenotype relationships collapsed down to 98 distinct genes once duplicate genes were excluded. The number of *P. bivittatus* genes associated with each phenotype is shown in Figure 3(a). The average percent identity for each phenotype is shown in Figure 3(b) and the standard deviation for the average percent identity within each phenotype is shown in Figure 3(c).

Genes in each of the four mature sperm phenotypes gene sets (sperm number [7 genes], sperm motility [12 genes], sperm physiology [7 genes], and capacitation [3 genes]) were analyzed for overlap across the phenotypes (Figure 4(a)). A total of 23 unique genes were distributed among the phenotypes with 7 genes being exclusive to sperm motility; an additional 7 genes were unique to the sperm number phenotype, 3 genes were specific to sperm physiology, and a single gene was associated with capacitation. Three genes were common between sperm motility and sperm physiology while just a single gene was found to be associated with both motility and capacitation. Interestingly, one gene was associated with the motility, physiology, and capacitation phenotypes. The majority of genes were unique to specific phenotypes.

Genes in each of the three abnormal morphological phenotypes associated with spermatogonia [8 genes], spermatocytes [21 genes], and spermatids [28 genes] were analyzed for overlap across the distinct phenotypes (Figure 4(b)). A total of 45 genes were distributed among the phenotypes with 22 unique to spermatids, 11 unique to spermatocytes, and just 2 genes unique to spermatogonia. Four genes were common among spermatids and spermatocytes while another four genes were common spermatocytes and spermatogonia. Only two genes were associated with all three phenotypes.

3.4. Functional Analysis of Sperm Phenotype-Specific Gene Sets Using Gene Ontology Annotation. Gene ontology (GO) enrichment was performed to assess the biological role of the sperm associated python genes (Table 2). Among biological process annotation, highly significant terms were identified relating to reproduction including “gamete generation”, “spermatogenesis”, “germ cell development”, “spermatid differentiation”, and “meiosis”. Many of these terms were associated with p values as low as 7.20E – 40 and 7.70E – 37. Among the enriched GO terms representing cellular component information were cilium, cell projection, acrosomal vesicle, and microtubule cytoskeleton. Within the molecular function GO terms enriched themes of transcriptional factor regulation and DNA binding were identified as well as ATP binding and kinase activity. The complete set of GO annotation data, including a list of genes enriched for each identified GO annotation term, is available in Supplemental File 3 (biological process), Supplemental File 4 (cellular compartment), and Supplemental File 5 (molecular function). A two-dimensional semantic scatter plot was generated from the gene ontology biological process annotation (Figure 5) in order to facilitate visualization of the GO enrichment data. Semantic relationships within the gene ontology annotation terms provide evidence of common themes relating to spermatogenesis and sperm motility and function in the context of reproduction.

3.5. KEGG Pathways Enriched for *P. bivittatus* Genes Associated with Sperm Phenotypes. KEGG pathways enriched for genes within the set of 98 *Python bivittatus* genes associated with sperm phenotypes were identified. Six pathways were
### Table 2: Gene ontology enrichment.

| Category                        | GO identifier | Annotation term                          | Count | Enrichment    | p value     |
|---------------------------------|---------------|------------------------------------------|-------|---------------|-------------|
| Biological process              | GO:0019953    | Sexual reproduction                       | 45    | 13.99         | 7.20E - 40  |
| Biological process              | GO:0007276    | Gamete generation                        | 41    | 14.78         | 7.70E - 37  |
| Biological process              | GO:0048232    | Male gamete generation                   | 37    | 17.11         | 3.46E - 35  |
| Biological process              | GO:0007283    | Spermatogenesis                           | 37    | 17.11         | 3.46E - 35  |
| Biological process              | GO:0032504    | Multicellular organism reproduction       | 42    | 12.28         | 1.54E - 34  |
| Biological process              | GO:0048609    | Reproductive process in a multicellular organism | 42    | 12.28         | 1.54E - 34  |
| Biological process              | GO:0003006    | Reproductive developmental process        | 27    | 14.67         | 2.70E - 23  |
| Biological process              | GO:0048610    | Reproductive cellular process             | 22    | 19.34         | 2.55E - 21  |
| Biological process              | GO:0007281    | Germ cell development                     | 18    | 25.38         | 2.20E - 19  |
| Biological process              | GO:0048515    | Spermatid differentiation                 | 14    | 34.98         | 6.97E - 15  |
| Biological process              | GO:0007286    | Spermatid development                     | 13    | 34.28         | 1.62E - 15  |
| Biological process              | GO:0007548    | Sex differentiation                       | 15    | 25.38         | 2.05E - 12  |
| Biological process              | GO:0045137    | Development of primary sexual characteristics | 12    | 13.46         | 1.15E - 09  |
| Biological process              | GO:0008406    | Gonad development                         | 11    | 13.99         | 5.09E - 09  |
| Biological process              | GO:0048608    | Reproductive structure development        | 11    | 12.43         | 1.60E - 08  |
| Biological process              | GO:0046661    | Male sex differentiation                  | 9     | 17.56         | 3.73E - 08  |
| Biological process              | GO:0051327    | M phase of meiotic cell cycle             | 9     | 13.08         | 3.79E - 07  |
| Biological process              | GO:0007126    | Meiosis                                   | 9     | 13.08         | 3.79E - 07  |
| Biological process              | GO:0051321    | Meiotic cell cycle                        | 9     | 12.82         | 4.43E - 07  |
| Biological process              | GO:0046546    | Development of primary male sexual characters | 7    | 15.34         | 5.69E - 06  |
| Biological process              | GO:0009566    | Fertilization                             | 7     | 12.62         | 1.77E - 05  |
| Biological process              | GO:0008584    | Male gonad development                    | 6     | 16.43         | 2.92E - 05  |
| Cellular component              | GO:0003929    | Cilium                                    | 7     | 8.89          | 1.24E - 04  |
| Cellular component              | GO:0031514    | Motile secondary cilium                   | 3     | 122.90        | 2.13E - 04  |
| Cellular component              | GO:0042995    | Cell projection                           | 14    | 3.29          | 2.44E - 04  |
| Cellular component              | GO:0001669    | Acrosomal vesicle                         | 4     | 17.72         | 1.41E - 03  |
| Cellular component              | GO:0019861    | Flagellum                                 | 4     | 14.25         | 2.65E - 03  |
| Cellular component              | GO:0030141    | Secretory granule                         | 6     | 5.46          | 4.57E - 03  |
| Cellular component              | GO:0016023    | Cytoplasmic membrane-bounded vesicle      | 10    | 2.98          | 5.65E - 03  |
| Cellular component              | GO:0031988    | Membrane-bounded vesicle                  | 10    | 2.89          | 6.93E - 03  |
| Cellular component              | GO:0005625    | Soluble fraction                          | 7     | 3.66          | 1.14E - 02  |
| Cellular component              | GO:0031410    | Cytoplasmic vesicle                       | 10    | 2.55          | 1.47E - 02  |
| Cellular component              | GO:0015630    | Microtubule cytoskeleton                  | 9     | 2.69          | 1.72E - 02  |
| Cellular component              | GO:0031982    | Vesicle                                   | 10    | 2.45          | 1.89E - 02  |
| Cellular component              | GO:0000267    | Cell fraction                             | 13    | 1.97          | 2.79E - 02  |
| Cellular component              | GO:0033391    | Chromatoid body                           | 2     | 54.62         | 3.56E - 02  |
| Cellular component              | GO:0060293    | Germ plasm                                | 2     | 46.82         | 4.14E - 02  |
| Cellular component              | GO:0045495    | Pole plasm                                | 2     | 46.82         | 4.14E - 02  |
| Cellular component              | GO:0043186    | P granule                                 | 2     | 46.82         | 4.14E - 02  |
| Cellular component              | GO:0034464    | BBSome                                    | 2     | 46.82         | 4.14E - 02  |
| Cellular component              | GO:0060170    | Cilium membrane                           | 2     | 40.97         | 4.72E - 02  |
| Molecular function              | GO:0046983    | Protein dimerization activity             | 13    | 3.71          | 1.60E - 04  |
| Molecular function              | GO:0042802    | Identical protein binding                 | 14    | 3.38          | 1.96E - 04  |
| Molecular function              | GO:0043565    | Sequence-specific DNA binding             | 12    | 3.06          | 1.61E - 03  |
| Molecular function              | GO:0003707    | Steroid hormone receptor activity         | 4     | 12.62         | 3.76E - 03  |
| Molecular function              | GO:0015631    | Tubulin binding                           | 5     | 7.73          | 3.82E - 03  |
| Molecular function              | GO:0030554    | Adenyl nucleotide binding                 | 20    | 1.96          | 4.37E - 03  |
| Molecular function              | GO:0001883    | Purine nucleoside binding                 | 20    | 1.93          | 5.16E - 03  |
| Molecular function              | GO:0042803    | Protein homodimerization activity         | 8     | 3.70          | 5.46E - 03  |
| Molecular function              | GO:0001882    | Nucleoside binding                        | 20    | 1.92          | 5.55E - 03  |
identified (Table 3). Among the 33 gene-pathway relationships identified were ten genes associated with pathways in cancer \((p \text{ value} = 1.64E-03)\), four genes implicated in p53 signaling \((p \text{ value} = 2.05E-02)\), and six genes enriched for cytokine-cytokine receptor interactions \((p \text{ value} = 7.38E-02)\). Although some of the results were associated with \(p\) values slightly larger than 0.05, they were still included in the table because the fold enrichment was greater than 2.5 for each enriched pathway, which provides supplemental support for their inclusion as they offer insight into the cellular and molecular processes underlying sperm differentiation, activation, and function in the python.

| Pathway-ID | Pathway name               | Count | Genes                                      | Enrichment | \(p\) value |
|------------|----------------------------|-------|--------------------------------------------|------------|-------------|
| hsa05200   | Pathways in cancer         | 10    | LAMA2, FOS, AR, PDGFA, RXRB, BAX, NOS2, KIT, FAS, CCNA1 | 3.45       | 1.64E-03    |
| hsa04115   | p53 signaling pathway      | 4     | BAX, APAFI, FAS, ATM                       | 6.65       | 2.05E-02    |
| hsa05222   | Small cell lung cancer     | 4     | LAMA2, RXRB, APAFI, NOS2                   | 5.38       | 3.54E-02    |
| hsa04210   | Apoptosis                  | 4     | BAX, APAFI, FAS, ATM                       | 5.20       | 3.87E-02    |
| hsa04202   | Calcium signaling pathway  | 5     | ATP2B4, CAMK4, PLCD4, NOS2, BDKRB2        | 3.21       | 6.44E-02    |
| hsa04060   | Cytokine-cytokine interaction | 6    | LEP, AMHR2, AMH, PDGFA, KIT, FAS           | 2.59       | 7.38E-02    |

Identification of KEGG pathways enriched for genes within *Python bivittatus* genes associated with sperm phenotypes. KEGG pathways were identified using the bioinformatics resource DAVID. The KEGG pathway identifier and pathway name are provided along with the number of genes identified in the pathway, the gene symbol for each identified gene, the fold enrichment, and the associated \(p\) value.

4. Discussion

The discovery of python genes associated with sperm phenotypes provides a tremendously important genetic resource for future use in studying reproduction and fertility in endangered and invasive reptile species. The results reported here highlight the value of comparative genomics and its application in species for which genomic resources are available. Through the mapping of python genes to specific phenotypes, it is now possible to develop more focused genetic research projects aimed at identifying genes associated with poor male fertility and reproductive success in endangered populations.

Our analysis of nucleotide and protein sequence similarity between *P. regius* and *P. bivittatus* provides evidence of similarity ranging from 86% to 98% at the nucleotide level and even higher when considering similarity at the protein level. Subsequently each of these two species can serve as a model organism for the other. For example, the genomic resources available for *P. bivittatus* can be used as a model for *P. regius*, such as for applications like PCR primer design. Housley et al. assessed PCR success among cross-species PCR primers and identified the relatedness of the target species and index species as one of the most important factors underlying PCR success [37]. Similarly, genetic dissection of *P. regius* phenotypes can be leveraged for applications in *P. bivittatus*. Unlike *P. bivittatus*, *P. regius* is a much smaller and
Figure 4: (a) Venn diagram illustrating relationship of genes among phenotypes associated with mature sperm. Genes in each of the four mature sperm phenotypes gene sets (sperm number [7 genes], sperm motility [12 genes], sperm physiology [7 genes], and capacitation [3 genes]) were analyzed for overlap across the phenotypes. A total of 23 unique genes were distributed among the phenotypes with 7 genes being exclusive to sperm motility, an additional 7 genes were unique to the sperm number phenotype, 3 genes were specific to sperm physiology, and just a single gene was only associated with capacitation. Three genes were common between sperm motility and sperm physiology while just a single gene was found to be associated with both motility and capacitation. Interestingly, one gene was associated with the motility, physiology, and capacitation phenotypes. The majority of genes were unique to specific phenotypes. (b) Venn diagram illustrating relationship of genes among phenotypes associated with morphological phenotypes in sperm precursors. Genes in each of the three abnormal morphological phenotypes associated with spermatogonia [8 genes], spermatocytes [21 genes], and spermatids [28 genes] were analyzed for overlap across the distinct phenotypes. A total of 45 genes were distributed among the phenotypes with 22 unique to spermatids, 11 unique to spermatocytes, and just 2 genes unique to spermatogonia. Four genes were common among spermatids and spermatocytes while another four genes were common spermatocytes and spermatogonia. Only two genes were associated with all three phenotypes.

more docile species which is amenable to reproductive studies as they are easily maintained in captivity and are known for being easy to breed in captivity.

The protein sequences selected to create alignments and phylogenetic trees were based on the limiting number of P. regius sequences available in NCBI (fewer than 30 protein sequences). Nonetheless, those selected were all implicated in sperm physiology or related to sperm biology. For example, the mitochondrial protein cytochrome b has been associated with decreased sperm mobility in association with specific haplotypes [58] and mutations in cytochrome b have been linked with asthenospermia [59]. Interestingly, Chen et al. demonstrated that cytochrome b is differentially expressed in X-chromosome versus Y-chromosome containing sperm [60].

The gene encoding epididymal protein E1 participates in sperm physiology and is associated with important sperm parameters. Giacomini et al. identified a threefold decrease in the expression of epididymal protein E1 in oligoasthenozoospermia compared to normozoospermia [61]. Epididymal protein E1 has been identified as a seminal plasma protein across species, such as boars [39] and bulls [62]. Moreover, a study in rams identified this protein as a factor that when added to frozen/thawed semen increased motility through repair of sperm damage that occurred during the cryopreservation process [63]. This protein exhibited marked conservation across taxa in our data. Specifically, it was conserved across mammals, birds, fish, reptiles, and even insects.

The protein cathepsin D is also involved in sperm biology and appears to play a role in its maturation. In mice, cathepsin D expression has been observed in the testis and cathepsin D has been detected on the surface of mouse sperm during epididymal maturation [48]. Similar results have been observed in humans. For example, cathepsin D expression has been observed in human Sertoli cells and Leydig cells and was shown to be anchored to the sperm surface in the postacrosomal region [64]. Evidence that cathepsin D may exhibit an evolutionarily conserved role in sperm physiology comes from a recent study in which
nodes indicate more significant term node. Blue nodes indicate less significant

When making bioinformatics predictions, one must always consider the trade-off that exists between false negatives and the proportion of true positives is very high, compared to a large number of predictions for which the false positive rate is high. When making bioinformatics predictions, one must always consider the trade-off that exists between false negatives and false positives [66, 67]. In our particular case, we wanted to provide a public resource that can facilitate efforts aimed at elucidating reptile reproduction. Since the time and cost of validating bioinformatics predictions in the laboratory are proportional to the number of predictions made, we chose to maximize the number of true positives and subsequently minimize the laboratory cost per true positive identified. The complete set of publicly released data is available in the form of six supplemental files associated with this study.

Although this work leverages bioinformatics and comparative genomics approaches, the genes identified are merely predicted to have sperm related phenotypes in the python species. As evidenced by the phylogenetic analysis performed as part of this study, protein sequences can diverge greatly across taxa. Using mouse genomic annotation to characterize reptile reproductive biology is a challenging process. Nonetheless, the identification of these genes is exciting and provides new avenues of subsequent investigation. However, one must proceed cautiously as it is likely some of the gene-phenotype associations we report may not be present in reptiles. Since it is well known that the reproductive biology of reptiles differs from mammals, the true value of this gene set and the accuracy of the functional predictions will require further study. Even so, the results obtained provide an important first step in expanding reproductive genomics to pythons. Future investigations into these ecologically important species will undoubtedly elucidate a variety of conserved and divergent reproductive properties between reptiles and mammals. Perhaps, some of these discoveries may offer novel pharmacological targets for developing novel reproductive technologies, not only in reptiles, but also in mammals.

5. Conclusion

To explore the possibility that *P. regius* and *P. bivittatus* exhibit sufficient genetic similarity to justify using one species as a genetic model for the other species, we assessed the level of sequence identity among a set of protein coding sequences, mitochondrial DNA sequences, and protein sequences between *Python bivittatus* (Burmese python) and *P. regius* (ball python) to assess the extent of sequence identity between the two species in genes implicated in sperm maturation and function. Alignments of epididymal protein E1 demonstrated the molecular similarity between these species with 98% identity when comparing the mRNA and 98% identity when comparing the protein sequences. In multiple sequence alignments and phylogenetic analysis we identify specific patterns of sequence similarity in epididymal protein E1 and cytochrome b which support the use of *P. regius* as a model for *P. bivittatus* and other snakes. Most importantly, we employed a comparative genomics strategy to identify python genes enriched for association with sperm related phenotypes. Our approach identified 129 gene-phenotype relationships corresponding to 98 unique genes representing 10 specific sperm associated phenotypes. We characterized these genes using gene ontology enrichment to annotate the biological processes, cellular compartments, and molecular functions associated with these reproductively important python genes. Our analysis also identified KEGG

cathepsin D was identified as a seminal plasma protein in carp [65].

Based on our multiple sequence alignments across taxa, proteins we investigated each exhibited unique patterns of conservation and divergence across the species. On the one hand, the conservation suggests that these factors have been conserved for millions of years to maintain their specific role in male reproduction. On the other hand, the distinct patterns of divergence between snakes and other reptiles, and even between reptiles and mammals, suggest that the biology may vary between different groups of taxa. Subsequently it is of value to expand our knowledge of the molecular basis of reproduction in reptiles and in particular snakes that can serve as research models for both endangered and invasive species.

In an attempt to expand molecular and genetic knowledge in reptile reproductive biology, we have leveraged a comparative genomics approach to identify *P. bivittatus* genes likely to be associated with sperm phenotypes. Although there are hundreds of genes implicated in reproductive biology, we specifically chose to limit our effort to just those for which each single gene we identified has been previously shown to cause a sperm phenotype in the mouse.

Our rationale for such stringency in our approach was that we prefer to identify a small set of genes, for which the proportion of true positives is very high, compared to a large number of predictions for which the false positive rate is high. When making bioinformatics predictions, one must always consider the trade-off that exists between false negatives and...
pathways enriched for these genes. Specifically, these genes are involved in the regulation of cell cycle, apoptosis, cancer, cytokine–cytokine signaling, and calcium regulation. To our knowledge, our results provide the first comprehensive view of genes associated with sperm development, sperm morphology, and sperm function in pythons. By making our data sets and findings publicly available, in the form of six supplemental files, we hope to facilitate the elucidation of reptile reproduction and promote effective conservation and management of reptiles worldwide.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

This work was supported by Western University of Health Sciences IMR Fund 12349V, Developing Resources for Reptilian Genomics. The authors wish to thank Dr. Steven Henriksen for his support of this project and the resources he has contributed to facilitate its success. The authors would also like to acknowledge the success of this project in spite of the obstacles. Most importantly, the authors wish to acknowledge Castoe TA, de Koning AP, Hall KT, Card DC, Schield DR, Fujita MK, Ruggiero RP, Degner JF, Daza JM, Gu W, Reyes-Velasco J, Shaney KJ, Castoe JM, Fox SE, Poole AW, Polanco D, Dobry J, Vandewege MW, Li Q, Schott RK, Kapusta A, Minx P, Feschotte C, Uetz P, Ray DA, Hoffmann FG, Bogden R, Smith EN, Chang BS, Vonk FJ, Casewell NR, Henkel CV, Richardson MK, Mackessy SP, Bronikowski AM, Yandell M, Warren WC, Secor SM, and Pollock DD for sequencing the *Python bivittatus* genome and making the sequences publicly available to the research community at large. Without their hard work and contribution to reptile genomics, their project would not be possible.

References

[1] T. A. Castoe, A. J. de Koning, K. T. Hall et al., “Sequencing the genome of the Burmese python (*Python molurus bivittatus*) as a model for studying extreme adaptations in snakes,” *Genome Biology*, vol. 12, no. 7, article no. 406, 2011.

[2] C. E. Wall, S. Cozza, C. A. Riquelme et al., “Whole transcriptome analysis of the fasting and fed Burmese python heart: insights into extreme physiological cardiac adaptation,” *Physiological Genomics*, vol. 43, no. 2, pp. 69–76, 2011.

[3] A. L. Andrew, D. C. Card, R. P. Ruggiero et al., “Rapid changes in gene expression direct rapid shifts in intestinal form and function in the Burmese python after feeding,” *Physiological Genomics*, vol. 47, no. 5, pp. 147–157, 2015.

[4] T. A. Castoe, A. P. J. de Koning, K. T. Hall et al., “The Burmese python genome reveals the molecular basis for extreme adaptation in snakes,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 51, pp. 20645–20650, 2013.

[5] R. G. Harvey, M. L. Brien, Michael et al., “Burmese pythons in south Florida: scientific support for invasive species management,” Tech. Rep. WEC242, University of Florida, IFAS Extension, Gainesville, Fla, USA, 2008.

[6] R. N. Reed, J. D. Willson, G. H. Rodda, and M. E. Dorcas, “Ecological correlates of invasion impact for Burmese pythons in Florida,” *Integrative Zoology*, vol. 7, no. 3, pp. 254–270, 2012.

[7] R. W. Snow, M. L. Brien, S. Cherkiss, L. Wilkins, and F. J. Mazzotti, “Dietary habits of the *Burmese python*, *Python molurus bivittatus*, in Everglades National Park, Florida,” *Herpetological Bulletin*, vol. 101, pp. 5–7, 2007.

[8] R. W. Snow, K. L. Krysko, K. M. Enge, L. Oberhofer, A. Warren-Bradley, and L. Wilkins, “Introduced populations of Boa constrictor (Boidae) and *Python molurus bivittatus* (Pythonidae) in southern Florida,” in *The Biology of Boas and Pythons*, R. W. Henderson and R. Powell, Eds., pp. 416–438, Eagle Mountain Publishing, 2007.

[9] M. E. Dorcas, J. D. Willson, R. N. Reed et al., “Severe mammal declines coincide with proliferation of invasive Burmese pythons in Everglades National Park,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 7, pp. 2418–2422, 2012.

[10] A. J. Piaggio, R. M. Engeman, M. W. Hopken et al., “Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA,” *Molecular Ecology Resources*, vol. 14, no. 2, pp. 374–380, 2014.

[11] D. G. Chapple, A. Birkett, K. A. Miller, C. H. Daugherty, and D. M. Gleeson, “Phylogeography of the endangered Otago skink, Oligosoma otagense: population structure, hybridisation and genetic diversity in captive populations,” *PLoS ONE*, vol. 7, no. 4, Article ID e34599, 2012.

[12] S. A. Hussain, C. Lessard, and M. Anzar, “Quantification of damage at different stages of cryopreservation of endangered North American bison (*Bison bison*) semen and the effects of extender and freeze rate on post-thaw sperm quality,” *Animal Reproduction Science*, vol. 129, no. 3–4, pp. 171–179, 2011.

[13] M. J. Ruiz-Lopez, D. P. Evenson, G. Espeso, M. Gomendio, and E. R. S. Roldan, “High levels of DNA fragmentation in spermatozoa are associated with inbreeding and poor sperm quality in endangered ungulates,” *Biology of Reproduction*, vol. 83, no. 3, pp. 332–338, 2010.

[14] M. Madeddu, F. Berlinguer, M. Ledda et al., “ Ejaculate collection efficiency and post-thaw semen quality in wild-caught Griffon vultures from the Sardinian population,” *Reproductive Biology and Endocrinology*, vol. 7, article 18, 2009.

[15] K. J. Mattson, A. De Vries, S. M. McGuire, J. Krebs, E. E. Louis, and N. M. Loskutoff, “Successful artificial insemination in the corn snake, *Elaphe guttata*, using fresh and cooled semen,” *Zoo Biology*, vol. 26, no. 5, pp. 363–369, 2007.

[16] R. Shine, “Reproductive strategies in snakes,” *Proceedings of the Royal Society B: Biological Sciences*, vol. 270, no. 1519, pp. 995–1004, 2003.

[17] D. M. Sever and W. C. Hamlett, “Female sperm storage in vultures from the Sardinian population,” *Reproductive Biology of Vultures from the Sardinian Population*.

[18] R. L. Zacariotti, K. F. Grego, W. Fernandas, S. S. Sant’Anna, and M. A. de Barras Vaz Guimarães, “Semen collection and evaluation in free-ranging Brazilian rattlesnakes (*Crotalus durissus terri ficus*),” *Zoo Biology*, vol. 26, no. 2, pp. 155–160, 2007.
[19] M. Moshiri, F. Todehdeghian, and A. Shiravi, "Study of sperm reproductive parameters in mature zanjani viper," Cell Journal, vol. 16, no. 2, pp. 111–116, 2014.

[20] B. M. Fahrig, M. A. Mitchell, B. E. Eilts, and D. L. Paccamonti, "Characterization and stored semen of cown from corn snakes (Elaphe guttata)," Journal of Zoo and Wildlife Medicine, vol. 38, no. 1, pp. 7–12, 2007.

[21] D. E. Basset Jr., M. S. Boguski, F. Spencer, R. Reeves, M. Goebl, and P. Hieter, "Comparative genomics, genome cross-referencing and XREFdb," Trends in Genetics, vol. 11, no. 9, pp. 372–373, 1995.

[22] D. E. Basset Jr., M. S. Boguski, F. Spencer et al., "Genome cross-referencing and XREFdb: implications for the identification and analysis of genes mutated in human disease," Nature Genetics, vol. 15, no. 4, pp. 339–344, 1997.

[23] C. Suter-Crazzolara and G. Kurapkat, "An infrastructure for comparative genomics to functionally characterize genes and proteins," Genome Inform Ser Workshop Genome Inform, vol. 11, pp. 24–32, 2000.

[24] S. Oduru, J. L. Campbell, S. Karri, W. J. Hendry, S. A. Khan, and S. C. Williams, "Gene discovery in the hamster: a comparative genomics approach for gene annotation by sequencing of hamster testis cDNAs," BMC Genomics, vol. 4, no. 1, article 22, 2003.

[25] S. K. Hawthorne, G. Goodarzi, J. Bagarova et al., "Comparative genomics of the sperm mitochondria-associated cysteine-rich protein gene," Genomics, vol. 87, no. 3, pp. 382–391, 2006.

[26] L. Yang, X. Zhang, J. Chen et al., "ReCGiP, a database of reproduction candidate genes in pigs based on bibliomics," Reproductive Biology and Endocrinology, vol. 8, article 96, 2010.

[27] K. J. Irizarry, S. B. Malladi, X. Gao et al., "Sequencing and comparative genomic analysis of 1227 Felis catus cDNA sequences enriched for developmental, clinical and nutritional phenotypes," BMC Genomics, vol. 13, no. 1, article 31, 2012.

[28] P. G. Baker, C. A. Goble, S. Bechhofer, N. W. Paton, R. Stevens, and A. Brass, "An ontology for bioinformatics applications," Bioinformatics, vol. 15, no. 6, pp. 510–520, 1999.

[29] M. Ashburner, C. A. Ball, J. A. Blake et al., "Gene ontology: tool for the unification of biology. The Gene Ontology Consortium," Nature Genetics, vol. 25, no. 1, pp. 25–29, 2000.

[30] N. H. Shah and N. V. Fedoroff, "CLENCH: a program for calculating CLuster ENriCHment using the gene ontology," Bioinformatics, vol. 20, no. 7, pp. 1196–1197, 2004.

[31] X. Wang, S. Pyne, and I. Dinu, "Gene set enrichment analysis for multiple continuous phenotypes," BMC Bioinformatics, vol. 15, article 260, 2014.

[32] C. L. Smith and J. T. Eppig, "The mammalian phenotype ontology: enabling robust annotation and comparative analysis," Wiley Interdisciplinary Reviews: Systems Biology and Medicine, vol. 1, no. 3, pp. 390–399, 2009.

[33] P. N. Robinson and S. Mundlos, "The human phenotype ontology," Clinical Genetics, vol. 77, no. 6, pp. 525–534, 2010.

[34] T. F. Hayamizu, S. de Coronado, G. Fragoso, N. Sioutos, J. A. Kadin, and M. Ringwald, "The mouse-human anatomy ontology mapping project," Database, vol. 2012, Article ID bar066, 2012.

[35] M. Kanehisa, "The KEGG database," Novartis Foundation Symposium, vol. 247, pp. 91–252, 2002.

[36] K. F. Aoki-Kinoshita and M. Kanehisa, "Gene annotation and pathway mapping in KEGG," Methods in Molecular Biology, vol. 396, pp. 71–91, 2007.

[37] F. Hahne, A. Mehrle, D. Arlt, A. Poustka, S. Wiemann, and T. Beissbarth, "Extending pathways based on gene lists using InterPro domain signatures," BMC Bioinformatics, vol. 9, article 3, 2008.

[38] D. W. Huang, B. T. Sherman, and R. A. Lempicki, "Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources," Nature Protocols, vol. 4, no. 1, pp. 44–57, 2009.

[39] V. González-Cadavid, J. A. M. Martins, F. B. Moreno et al., "Seminal plasma proteins of adult boars and correlations with sperm parameters," Theriogenology, vol. 82, no. 5, pp. 697–707, 2014.

[40] R. J. Llanos, C. M. Whitacre, and D. C. Miceli, "Potential involvement of C1 complement factor in amphibian fertilization," Comparative Biochemistry and Physiology—Part A: Molecular and Integrative Physiology, vol. 127, no. 1, pp. 29–38, 2000.

[41] A. Asano, J. L. Nelson-Harrington, and A. J. Travis, "Phospholipase B is activated in response to steroid removal and stimulates acrosome exocytosis in murine sperm," The Journal of Biological Chemistry, vol. 288, no. 39, pp. 28104–28115, 2013.

[42] J. B. Atiken, N. Naumovski, B. Curry, C. G. Gruppen, Z. Gibb, and R. J. Atiken, "Characterization of an L-amino acid oxidase in equine spermatozoa," Biology of Reproduction, vol. 92, no. 5, article 125, 2015.

[43] V. G. Da Ros, M. W. Muñoz, M. A. Battistone et al., "From the epididymis to the egg: participation of CRISP proteins in mammalian fertilization," Asian Journal of Andrology, vol. 17, no. 5, pp. 711–715, 2015.

[44] N. Emami, A. Scorzis, A. Sosaapillai, T. Earle, B. Mullen, and E. P. Diamandis, "Association between kallikrein-related peptidases (KLKs) and macroscopic indicators of semen analysis: their relation to sperm motility," Biological Chemistry, vol. 390, no. 9, pp. 921–929, 2009.

[45] S. D. Carson and C. J. De Jonge, "Activation of coagulation factor X in human semen," Journal of Andrology, vol. 19, no. 3, pp. 289–294, 1998.

[46] Y.-T. Tseng, J.-Y. Hsia, C.-Y. Chen, N.-T. Lin, P. C.-S. Chong, and C.-Y. Yang, "Expression of the sperm fibrous sheath protein CABYR in human cancers and identification of α-enolase as an interacting partner of CABYR-a," Oncology Reports, vol. 25, no. 4, pp. 1169–1175, 2011.

[47] A. Gedakaew, N. Kosa, S. Siricoon, S. V. Grams, and R. Grams, "A 170 kDa multi-domain cystatin of Fasciola gigantica is active in the male reproductive system," Molecular and Biochemical Parasitology, vol. 196, no. 2, pp. 100–107, 2014.

[48] S. Asuvapongpatana, A. Saewu, C. Chotiwatthanakun, R. Vanichwiriyakit, and W. Weerachatanukul, "Localization of cathepsin D in mouse reproductive tissues and its acquisition onto sperm surface during epididymal sperm maturation," Acta Histochemica, vol. 115, no. 5, pp. 425–433, 2013.

[49] D. Selvi Rani, A. Vanniarajan, N. J. Gupta, B. Chakravarty, L. Singh, and K. Thangaraj, "A novel missense mutation C11994T in the mitochondrial ND4 gene as a cause of low sperm motility in the Indian subcontinent," Fertility and Sterility, vol. 86, no. 6, pp. 1783–1785, 2006.

[50] R. Zhou, R. Wang, Y. Qin et al., "Mitochondria-related miR-15a-5p reduces cellular ATP production by targeting CYTB in asthenozoospermia," Scientific Reports, vol. 5, Article ID 17743, 2015.

[51] G.-H. Mao, Y.-N. Wang, M. Xu, W.-L. Wang, L. Tan, and S.-B. Tao, "Polymorphisms in the MT-ATP6 and MT-CYB genes in..."
in vitro fertilization failure," *Mitochondrial DNA*, vol. 26, no. 1, pp. 20–24, 2015.

[52] D. W. Huang, B. T. Sherman, Q. Tan et al., "DAVID bioinformatics resources: expanded annotation database and novel algorithms to better extract biology from large gene lists," *Nucleic Acids Research*, vol. 35, no. 2, pp. W169–W175, 2007.

[53] G. Dennis Jr., B. T. Sherman, D. A. Hosack et al., "DAVID: database for annotation, visualization, and integrated discovery," *Genome Biology*, vol. 4, no. 5, article P3, 2003.

[54] M. Kanehisa, S. Goto, S. Kawashima, Y. Okuno, and M. Hattori, "The KEGG resource for deciphering the genome," *Nucleic Acids Research*, vol. 32, pp. D277–D280, 2004.

[55] B. T. Sherman, D. W. Huang, Q. Tan et al., "DAVID knowledgebase: a gene-centered database integrating heterogeneous gene annotation resources to facilitate high-throughput gene functional analysis," *BMC Bioinformatics*, vol. 8, article 426, 2007.

[56] X. Jiao, B. T. Sherman, D. W. Huang et al., "DAVID-WS: a stateful web service to facilitate gene/protein list analysis," *Bioinformatics*, vol. 28, no. 13, pp. 1805–1806, 2012.

[57] D. J. E. Houlsley, Z. A. Zalewski, S. E. Beckett, and P. J. Venta, "Design factors that influence PCR amplification success of cross-species primers among 1147 mammalian primer pairs," *BMC Genomics*, vol. 7, article 253, 2006.

[58] F. Montiel-Sosa, E. Ruiz-Pesini, J. A. Enríquez et al., "Differences of sperm motility in mitochondrial DNA haplogroup U sublineages," *Gene*, vol. 368, no. 1-2, pp. 21–27, 2006.

[59] C.-Q. Feng, Y.-B. Song, Y.-G. Zou, and X.-M. Mao, "Mutation of MTCYB and MTATP6 is associated with asthenospermia," *Zhonghua Nan Ke Xue*, vol. 14, no. 4, pp. 321–323, 2008.

[60] X. Chen, Y. Yue, Y. He et al., "Identification and characterization of genes differentially expressed in X and Y sperm using suppression subtractive hybridization and cDNA microarray," *Molecular Reproduction and Development*, vol. 81, no. 10, pp. 908–917, 2014.

[61] E. Giacomini, B. Ura, E. Giolo et al., "Comparative analysis of the seminal plasma proteomes of oligoasthenozoospermic and normozoospermic men," *Reproductive BioMedicine Online*, vol. 30, no. 5, pp. 522–531, 2015.

[62] J. P. A. Rego, J. M. Crisp, A. A. Moura et al., "Seminal plasma proteome of electroejaculated Bos indicus bulls," *Animal Reproduction Science*, vol. 148, no. 1-2, pp. 1-17, 2014.

[63] A. Bernardini, F. Hozbor, E. Sanchez, M. W. Fornés, R. H. Alberio, and A. Cesari, "Conserved ram seminal plasma proteins bind to the sperm membrane and repair cryopreservation damage," *Theriogenology*, vol. 76, no. 3, pp. 436–447, 2011.

[64] A. Saewu, S. Asuvapongpatana, C. Chotiwatthanakun, A. Tantiwongse, W. Weerachatyanukul, and S. Thitilertdecha, "Cathepsin D in human reproductive tissues: cellular localization in testis and epididymis and surface distribution in different sperm conditions," *Journal of Andrology*, vol. 33, no. 4, pp. 726–734, 2012.

[65] M. A. Dietrich, G. J. Arnold, J. Nynca, T. Fröhlich, K. Otte, and A. Ciereszko, "Characterization of carp seminal plasma proteome in relation to blood plasma," *Journal of Proteomics*, vol. 98, pp. 218–232, 2014.

[66] F. De Smet, Y. Moreau, K. Engelen, D. Timmerman, I. Vergote, and B. De Moor, "Balancing false positives and false negatives for the detection of differential expression in malignancies," *British Journal of Cancer*, vol. 91, no. 6, pp. 1160–1165, 2004.

[67] J. Wu, N. I. Lenchik, and I. C. Gerling, "Approaches to reduce false positives and false negatives in the analysis of microarray data: applications in type 1 diabetes research," *BMC Genomics*, vol. 9, supplement 2, article S12, 2008.