A phylogenomic evaluation of the relationships among herons (Aves: Ardeidae)

By

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ABSTRACT

Herons (Aves: Ardeidae) are a cosmopolitan family of birds that comprises ~65 species and is found on all continents except Antarctica. Despite being well-studied by ornithologists, phylogenetic relationships within the family are uncertain. For example, the earliest diverging lineages have not been confidently identified, and the monophyly of some genera has been questioned. Here, I present the results of a molecular phylogenetic analysis of herons that includes ~70 percent of species diversity. Analyses of thousands of genomic loci yielded a fully resolved and well-supported phylogeny for the herons. Phylogenetic relationships were broadly congruent across all analytical methods and clarified the composition and placement of several genera that have been traditionally difficult to place. For example, I identified the tiger-herons as the sister-group to all other herons and recovered non-monophyly for some tribes (Nycticoracini and Egrettini) and genera (e.g. *Gorsachius* and *Ixobrychus*).

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INTRODUCTION

Herons (Aves: Ardeidae) are a family of wading birds in the order Pelecaniformes (Hackett et al. 2008; Jarvis et al. 2014; Prum et al. 2015). The herons consist of 62-65 species, 17-19 genera and five subfamilies (Kushlan and Hancock 2005; Dickinson and Remsen 2013; Clements et al. 2017; Gill and Donsker 2018; Martínez-Vilalta et al. 2018). The subfamilies *sensu* Kushlan and Hancock (2005) are: (1) Ardeinae, the "typical herons" (*Ardea*, *Butorides*, *Ardeola*, *Egretta*, *Syrigma*, *Pilherodius*, *Nyctanassa, Nycticorax, Gorsachius*); (2) Botaurinae, the bitterns (*Botaurus*, *Ixobrychus*, *Zebrilus*); (3) Tigrisomatinae, the tiger-herons (*Tigrisoma*, *Tigriornis*, *Zonerodius*); (4) Agamiinae, the Agami Heron (*Agamia agami*); and (5) Cochleariinae, the Boat-billed Heron (*Cochlearius cochlearius*). Three tribes, all within the subfamily Ardeinae, are also recognized by Kushlan and Hancock (2005): (1) Ardeini (*Ardea, Butorides, Ardeola*); (2) Egrettini (*Egretta*, *Syrigma*, *Pilherodius*, *Nyctanassa*); and (3) Nycticoracini (*Nycticorax, Gorsachius*).

Taxonomy

I follow the specific and generic taxonomy proposed by Gill and Donsker (2018), given its recency of publication. Because Gill and Donsker (2018) do not include tribe or subfamily in their list, for higher-level taxonomy I follow the most recent suprageneric classification available, that of Kushlan and Hancock (2005) (Table 1). As a result, I retain *Dupetor* and *Bubulcus* as monotypic genera (*sensu* Gill and Donsker, 2018), despite their inclusion in *Ixobrychus* and *Ardea*, respectively, by Kushlan and Hancock (2005). Similarly, I recognize the White-backed Night-Heron *Gorsachius leuconotus*, not *Nycticorax leuconotus*, following

Kushlan and Hancock (2005). I also provide synonymies for generic names used herein (Table 2).

The backbone of the heron tree

Sheldon et al. (1995) highlighted the identification of the basal lineages of herons as one of the outstanding questions of higher-level phylogeny. Bock (1956) used morphological and ecological traits in a non-cladistic framework to propose that Botaurinae was the most primitive group, with Ardeinae being most derived. Payne and Risley (1976) used osteological characters to conduct the first cladistic study of herons. Payne and Risley (1976), in addition to recognizing the subfamily Tigrisomatinae, recovered Ardeinae as the least and Botaurinae as the most, derived subfamily. Sheldon (1987a; b) used DNA-DNA hybridization data and recovered Cochleariinae and Tigrisomatinae as sister taxa, with them in turn being sister to the rest of the herons (Figure 1). Sheldon et al. (1995) added two new samples to the Sheldon (1987b) DNA-DNA hybridization data set and recovered a conflicting topology to that recovered in Sheldon (1987a; b): Tigrisomatinae was recovered as sister to the rest of the herons, and Cochleariinae was recovered sister to Botaurinae and Ardeinae. These contradictory results prompted Sheldon et al. (1995) to conclude that support for the earliest diverging lineage was equivocal and suggested that the positions of Cochleariinae and Tigrisomatinae were best considered unresolved at the base of the tree. A cladistic analysis of vocalizations by McCracken and Sheldon (1997) supported the hypothesis that Cochleariinae and Tigrisomatinae were early diverging lineages, but the data were unable to resolve specific relationships for either group. Päckert et al. (2014) did not sample any members of Tigrisomatinae but recovered Cochleariinae as sister to the rest of the herons included in that study. A recent phylogeny reconstructed by

Huang et al. (2016) using mtDNA data from 32 species offers an alternative hypothesis. Surprisingly, they recovered *Zebrilus*, traditionally considered a member of Botaurinae, as sister to the rest of the herons. They also recovered Cochleariinae as sister to clade consisting of Ardeinae and Botaurinae. Tigrisomatinae was recovered as sister to clade consisting of Cochleariinae, Ardeinae and Botaurinae.

The sister relationship of Botaurinae and Ardeinae has not been as controversial. Although Bock (1956) considered the Botaurinae the most primitive group, most subsequent evaluations have suggested that Botaurinae is one of the more recently diverging lineages, and sister to the Ardeinae. All DNA-DNA hybridization studies (Sheldon 1987a; Sheldon 1987b; Sheldon and Kinnarney 1993; Sheldon et al. 1995), cladistic analysis of vocal characters (McCracken and Sheldon 1997), and multiple mtDNA studies (Sheldon et al. 2000; Chang et al. 2003; Zhou et al. 2014; Päckert et al. 2014; Zhou et al. 2016; Huang et al. 2016) have supported the sister relationship between Ardeinae and Botaurinae.

The position and composition of genera

Sheldon et al. (1995) highlighted the composition and position of enigmatic genera as the second outstanding question of higher-level phylogeny. In this category, they include: *Agamia, Ardeola, Pilherodius and Gorsachius.* In the intervening decades there has also been considerable disagreement surrounding the placement of *Nycticorax* and *Nyctanassa* and the composition of *Ixobrychus*.

Position of Agamia

The Agami Heron (*Agamia agami*) is a long and slender heron that breeds in colonies and occurs in Central and northern South America. Bock (1956) considered it as a member of Ardeinae, and this hypothesis was corroborated by Payne and Risley (1976) and Huang et al. (2016), who both recovered it as nested within this subfamily. Payne and Risley (1976) recovered it as sister to a group consisting of members of *Ardeola* and *Egretta*. Huang et al. (2016) recovered it as sister to *Ardeola*. Kushlan and Hancock (2005) cite personal communication with Kevin McCracken, who alternatively recovered *Agamia* outside of the Ardeinae in an unpublished phylogeny estimated from mtDNA data. It is for this reason that Kushlan and Hancock (2005) place the Agami Heron in its own subfamily.

Position and composition of Ardeola

Ardeola sensu Gill and Donsker (2018) includes 6 species, with its center of diversity in the Afrotropics. Colloquially referred to as the "pond herons", members of this genus are characterized by their stocky appearance and affinity for marshy habitats (Kushlan and Hancock 2005). Bock (1956) subsumed *Bubulcus* within *Ardeola*, whereas Curry-Lindahl (1971) questioned the placement of *Bubulcus* within *Ardeola*. Payne and Risley (1976) synonymized *Butorides* with *Ardeola,* while placing *Ardeola* within Ardeinae. Chang et al. (2003) conducted the first molecular phylogenetic analysis that included *Ardeola* and recovered *Ardeola bacchus* within Ardeinae but received equivocal support for its placement within the subfamily. Zhou et al. (2014; 2016) recovered *Ardeola* and *Butorides* as sister taxa, with that clade being sister to *Ardea*. Huang et al. (2016) recovered *Ardeola* as sister to *Agamia*, and *Butorides* as sister to *Gorsachius*. *Ardeola* is considered by Kushlan and Hancock (2005) as a member of Ardeini.

Position of Pilherodius

The Capped Heron (*Pilherodius pileatus*) is a medium-sized heron characterized by the presence of long plumes. Restricted to the Neotropics, *Pilherodius* inhabits forested aquatic habitats (Kushlan and Hancock 2005). Few molecular phylogenetic studies have included *Pilherodius*, and as such, its placement within the herons is still debated. *Pilherodius* was synonymized with the night-heron genus *Nycticorax* by Bock (1956). Curry-Lindahl (1971), conversely, suggested that *Pilherodius* was more closely related to *Ardeola*. Payne and Risley (1976) recovered *Pilherodius* as sister to *Ardea*. Huang et al. (2016) recovered *Pilherodius* as sister to the monotypic Whistling Heron (*Syrigma sibilatrix*), with them being sister to members of *Egretta*. Kushlan and Hancock (2005) consider *Pilherodius* a member of the Egrettini.

Position and composition of Gorsachius

Gorsachius sensu Gill and Donsker (2018) includes the White-eared Night-Heron *Gorsachius magnificus*, the White-backed Night-Heron *Gorsachius leuconotus*, the Malayan Night-Heron *Gorsachius melanolophus,* and the Japanese Night-Heron *Gorsachius goisagi*. All four species are solitary nesters, exhibit nocturnal behavior, and are poorly studied (Kushlan and Hancock 2005). Bock (1956) synonymized *Oroanassa magnificus* and *Calherodius leuconotus* with *Gorsachius.* Curry-Lindahl (1971) suggested equivocally that *Calherodius* is allied to either *Gorsachius* or *Nycticorax*. Payne and Risley (1976) synonymized *Oroanassa magnifica*, *Gorsachius goisagi* and *Gorsachius melanolophus* with *Nycticorax*. The first study to include molecular data for *Gorsachius* was Zhou et al. (2016), who recovered a group consisting of *Gorsachius goisagi* and *Gorsachius melanolophus* as sister to *Nycticorax*, albeit with low

bootstrap support (BS=51). *Gorsachius magnificus*, on the other hand, was recovered as sister to *Egretta*, rendering *Gorsachius* non-monophyletic. Huang et al. (2016), alternatively, recovered *Gorsachius goisagi* as sister to *Butorides*. Kushlan and Hancock (2005) consider *Gorsachius goisagi*, *Gorsachius melanolophus*, and *Gorsachius magnificus* as members of Nycticoracini.

Position and composition of Nycticorax and Nyctanassa

Nycticorax sensu Gill and Donsker (2018) includes two species, the Nankeen Night-Heron *Nycticorax caledonicus* and Black-crowned Night-Heron *Nycticorax nycticorax*. *Nyctanassa sensu* Gill and Donsker (2018) is monotypic and includes the Yellow-crowned Night-Heron *Nyctanassa violacea*. Bock (1956) considered *Pilherodius*, *Syrigma* and *Nyctanassa* all to be synonymous with *Nycticorax*. Curry-Lindahl (1971) also suggested *Nyctanassa* and *Nycticorax* were closely related to one another. Payne and Risley (1976) recovered *Nyctanassa* as sister to *Nycticorax* but maintained both genera. Sheldon (1987a) recovered *Nyctanassa* and *Nycticorax* as sister to each other but unresolved within Ardeinae. Sheldon (1987b) and McCracken and Sheldon (1997) did not recover *Nyctanassa* and *Nycticorax* as sister to each other, but rather recovered each as unresolved within the Ardeinae. Chang et al. (2003) also recovered equivocal support for the sister relationship between *Nycticorax* and *Nyctanassa*, with results of one analysis supporting this relationship and another leaving both genera unresolved within the Ardeinae. Zhou et al. (2014) recovered *Nycticorax* as sister to *Egretta*, although with poor support (BS=54). Zhou et al. (2016), as previously mentioned, recovered *Nycticorax* as sister to *Gorsachius goisagi* and *Gorsachius melanolophus* with low bootstrap support (BS=51). Huang et al. (2016) recovered *Nycticorax* as sister to a group

consisting of *Butorides* and *Gorsachius goisagi*. Kushlan and Hancock (2005) considered *Nyctanassa* as a member of the Egrettini and *Nycticorax* as a member of the Nycticoracini.

Composition of Ixobrychus

Ixobrychus is the most species-rich genus within Botaurinae. Found on all continents with the exception of Antarctica, members of this genus are characterized by their relatively small size and sexually dimorphic plumage (Kushlan and Hancock 2005). Bock (1956) recognized eight species of *Ixobrychus* and synonymized the Black Bittern *Dupetor flavicollis*, which had been recognized as such by Peters (1931), with *Ixobrychus*. Payne and Risley (1976) corroborated the classification of Peters (1931), recovering *Ixobrychus* as monophyletic. Sheldon (1987b) found the Least Bittern *Ixobrychus exilis* as more closely related to *Botaurus* than to other members of *Ixobrychus* (Figure 1). Päckert et al. 2014 carried out the most comprehensive molecular evaluation of *Ixobrychus* to date, sampling eight species of *Ixobrychus*. They recovered *Ixobrychus exilis* as sister to three species of *Botaurus* rather than other *Ixobrychus* species. The Stripe-backed Bittern *Ixobrychus involucris* was equivocally placed, with one analysis suggesting it was sister to *Ixobrychus exilis* and *Botaurus*, and another suggesting it was sister to other members of *Ixobrychus*.

Here, I infer phylogenetic relationships among herons by analyzing thousands of loci from ~70 % of all currently recognized species, 18 genera, and three closely-related outgroup species. Specifically, my data resolve long-standing controversies in heron systematics, such as the identification of the earliest-diverging lineages and the position and composition of some genera

that have been either been difficult to place, have been hypothesized to be non-monophyletic, or both.

MATERIALS AND METHODS

Sampling and Sample Preparation

I extracted DNA from fresh muscle tissue using the Qiagen ® DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) and the Maxwell ® RSC Blood DNA kit (Promega, Fitchburg, WI), following the manufacturers' protocols. Taxon sampling included 45 heron species and three outgroup taxa (Table 3), *Plegadis*, *Pelecanus* and *Balaeniceps,* chosen because of close relationships to Ardeidae in several recent molecular studies (Hackett et al. 2008; Jarvis et al. 2014; Prum et al. 2015). I then quantified extracts using a Qubit 2.0 Fluorometer (Life Technologies) using a Qubit ® dsDNA BR Assay Kit following manufacturer's protocol and standardized each extract to a concentration of 5 ng/μL.

UCE Library Preparation

I fragmented 250 ng of DNA from each sample using a Covaris S220 focus-ultrasonicator with the following settings: 175 W peak incident power, a duty factor of 2%, and 200 cycles per bust for 44-45 seconds. I targeted fragments of 500-600 bp in length for each sample. I then prepared libraries using Kapa Biosystems Library Hyper Prep Kits (KBLPK; Kit # KK2602). Each sample was then subjected to end repair and A-tailing on a thermal cycler, followed by the ligation of two universal iTru stubs (iTru Stub Oligo 1: 5' – /5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCA*C – 3', iTru Stub Oligo 2:5' – ACACTCTTTCCCTACACGACGCTCTTCCGATC*T – 3'). I purified samples with an

Agencourt AMPure XP bead cleanup (0.8x) subsequent to adapter ligation. I amplified libraries using 10 PCR cycles and then pooled them, including 8 libraries per pool. I subjected amplified pooled libraries to a 1× Agencourt AMPure XP bead cleanup, and subsequently quantified them using a Qubit 2.0 Fluorometer (Life Technologies) using a Qubit® dsDNA HS Assay Kit. I enriched pooled libraries for 5,060 UCE loci using 5,742 baits in the MYbaits Tetrapods-UCE-5kv1-96 library capture kit (sequences available at ultraconserved.org) (Faircloth et al. 2012). I then performed the enrichment reaction using the following steps: (1) each pooled library was placed in the thermal cycler at 95°C for 5 minutes; (2) the hybridization mix (including baits) and each pooled library was placed in the thermal cycler at 65°C for 5 minutes, in separate strip tubes; (3) each pooled library was combined with the hybridization mix (including baits) into one strip tube, homogenized, and placed in the thermal cycler at 65°C for 24 hours (subsequently referred to as capture reactions). I then mixed pre-washed Dynabeads® MyOneTM Straptavidin C1 beads with the capture reactions and incubated on a heated block at 65°C for 30 minutes. Subsequently, I washed libraries with a buffer consisting of 1200 µL of nano-filtrated water, 300 µL of buffer 2 (in MYbaits kit), and 12.12 µL of HYB 4 (in MYbaits kit), per capture reaction. Following the wash, I resuspended libraries using 30 µL of 10 mM Tris-Cl, 0.05% TWEEN®-20 solution, which consisted of 1500 µL of 10 nM Tris-Cl and 0.75 µL of TWEEN®-20. I subsequently amplified capture-reaction products using 18 PCR cycles, followed by a 1.2x Agencourt AMPure XP bead cleanup. Lastly, I quantified enriched, pooled libraries using a Qubit 2.0 Fluorometer (Life Technologies) using a Qubit® dsDNA HS Assay Kit.

Sequencing

I pooled the 48 dual-indexed samples with samples from other projects (N=120) on a single lane of an Illumina HiSeq 3000 flow cell at the Oklahoma Medical Research Foundation and generated 150bp paired-end reads (Oklahoma City, OK).

Data Assembly

I trimmed low-quality bases and adapter sequences from reads using illumiprocessor 2.0.6 (Faircloth 2013), which incorporates trimmomatic (Lohse et al. 2012; Del Fabbro et al. 2013). Contigs for each individual were assembled using SPAdes 3.11.1 (Bankevich et al. 2012). I used several modules from the python package PHYLUCE 1.5.0 (Faircloth 2015) for UCE processing and analysis. I used the module *phyluce_assembly_match_contigs_to_probes* to extract contigs that matched UCE loci. I compiled two data sets: a complete data set that included UCE loci present in all 48 taxa and an incomplete data set that included UCE loci present in at least 75% of taxa, using the *phyluce_assembly_get_fastas_from_match_counts* module. I aligned UCE loci using the *phyluce_align_seqcap_align* module, which incorporates MAFFT 7.13 (Katoh and Standley 2013). I did not trim nucleotides from the alignment ends during this step. Rather, I trimmed alignments using Gblocks 0.91 (Castresana 2000) using default parameters, with the exception of the minimum number of sequences for a flank position, which was set at 65% of taxa. I obtained summary statistics of contig and UCE length and coverage (Table 1) using the *phyluce_assembly_get_trinity_coverage* and the *phyluce_assembly_get_trinity_coverage_for_uce_loci* modules.

Phylogenetic analyses

I used the maximum likelihood (ML) method RAxML 8.2.11 (Stamatakis 2014) to estimate species trees from concatenated matrices of both the complete (1,254 loci) and incomplete (4,602 loci) data sets, assuming a general time reversible model of rate substitution and gamma-distributed rates among sites. I assumed this model of sequencing evolution because of its wide use in similar studies using UCEs (e.g., Moyle et al. 2016, Andersen et al. 2017). I assessed support for trees from both alignments using 1000 rapid bootstrap replicates.

Species trees estimated using concatenated alignments can result in incorrect and highly supported trees (Kubatko and Degnan 2007), so I also conducted coalescent-based species-tree analyses. I inferred species trees using SVDQuartets (Chifman and Kubatko 2014) using the program PAUP* v4a159 (Swofford 2003). I assessed support for these phylogenies using 100 bootstraps. SVDQuartets analyzes quartets of species using singular value decomposition of the matrix of site pattern frequencies and assembles a species tree from the resulting quartets. Subsequently, I estimated gene trees for each locus in the incomplete data set (4,602 loci) in RAxML 8.2.11 (Stamatakis 2014), using 10 independent runs. Nodal support for gene trees were assessed with 500 bootstrap replicates. I used these gene trees as input for ASTRAL 4.10.12 (Mirarab et al. 2014). I assessed support for this phylogeny using 100 multilocus bootstraps. Multi-locus bootstrapping resamples sites within a locus and loci within a data set (Seo 2008). Although ASTRAL is not strictly considered a coalescent method, it is statistically consistent with the multispecies coalescent model (Liu and Yu 2010).

RESULTS

Molecular data summary statistics

The mean number of UCE loci recovered per sample (Table 3) was 4581.5. The maximum number of loci was 4761 (*Ixobrychus cinnamomeus*) and the minimum number was 3538 (*Nyctanassa violacea*). The mean depth of coverage for UCE loci was 72.5x, with a minimum of 1.3x (*Ixobrychus eruhythmus*) and a maximum of 162.6x (*Ixobrychus sinensis*). Before trimming, the mean UCE contig length was 1122.3 bp, with a minimum of 764.1 bp (*Agamia agami*) and a maximum of 1338.4 bp (*Balaeniceps rex*).

The complete UCE matrix consisted of 48 taxa, 1,254 loci and 1,429,392 bp, whereas the incomplete (75%) matrix consisted of 48 taxa, 4,602 loci, and 4,932,275 bp.

Topologies and nodal support

All analyses produced topologies that were broadly congruent with one another (Figures 2-6). The only relationship that differed among analyses was that of *Egretta thula* in the SVDQuartets analysis of the incomplete data set (Figure 4). A majority of nodes received 100% bootstrap support across all five analyses. A few received less than 90 % bootstrap support, and these included: (1) the sister relationship of the Tigrisomatinae to the rest of herons in both SVDQuartets analyses (Figures 4 and 5); (2) the sister relationship of the Ardeini and Egrettini in the RAxML analysis of the complete data set (Figure 2); (3) the placement of *Egretta thula* in both SVDQuartets analyses (Figures 4 and 5); (4) the sister relationship of *Nyctanassa* to *Nycticorax* in the SVDQuartets analysis of the incomplete data set (Figure 4); and (5) the sister relationship of *Ardea alba* and *Ardea intermedia* in the SVDQuartets analysis of the incomplete data set (Figure 4).

Relationships among subfamilies

All analyses produced identical relationships among subfamilies. The herons were recovered as monophyletic (Figures 2-6), with the Tigrisomatinae as sister to the rest of the herons. The support for this relationship was mixed: SVDQuartets analyses poorly supported this relationship (50% bootstrap support), whereas all other analyses fully supported this relationship (100% bootstrap support). Cochleariinae was recovered as sister to a clade consisting of Agamiinae, Ardeinae and Botaurinae. Ardeinae and Botaurinae were recovered as sister to each other. The placement of Cochleariinae and Agamiinae received 100% bootstrap support of 100% across all analyses, along with the sister relationship of the Botaurinae and Ardeinae (Figures 2-6).

Relationships within Tigrisomatinae

All analyses identified the Tigrisomatinae as monophyletic and produced congruent topologies of the group, with full bootstrap support (Figures 2-6). *Tigriornis* was recovered as sister to *Tigrisoma*. Within *Tigrisoma*, *Tigrisoma fasciatum* and *Tigrisoma mexicanum* were recovered as sister, with that clade recovered as sister to *Tigrisoma lineatum*.

Relationships within Botaurinae

All analyses identified the Botaurinae as monophyletic and produced congruent topologies within the group (Figures 2-6). *Zebrilus* was recovered as sister to two clades of *Ixobrychus sensu lato*. The first included *Ixobrychus involucris* as sister to group consisting of *Ixobrychus exilis* as sister to a group consisting of *Botaurus poiciloptilus* and *Botaurus lentiginosus*. The second clade included two groups, one comprising *Dupetor flavicollis* as sister to a group

consisting of *Ixobrychus eruhythmus* and *Ixobrychus cinnamomeus*, the second including *Ixobrychus sturmii* as sister to a group consisting of *Ixobrychus sinensis* and *Ixobrychus dubius*. All nodes within the Botaurinae were fully supported across all analyses (Figures 2-6).

Relationships within Ardeinae

Ardeinae *sensu* Kushlan and Hancock (2005) was recovered as monophyletic. All analyses also produced congruent topologies for the group (Figures 2-6). *Gorsachius melanolophus* was recovered as sister to the rest of the Ardeinae. Within the Ardeinae, Egrettini was recovered as sister to a group consisting of the Ardeini, *Nycticorax* and *Nyctanassa*. This relationship, however, did not always receive 100 % bootstrap support, receiving a bootstrap value of 94% in the ASTRAL analysis (Figure 6), a bootstrap support value of 95% in the RAxML analysis of the incomplete data set (Figure 3), and bootstrap support of 82% in the RAxML analysis of the complete data set (Figure 2).

Gorsachius was recovered as polyphyletic in all five analyses. As previously mentioned, *Gorsachius melanolophus* was recovered as sister to the rest of the Ardeinae. *Gorsachius leuconotus* was recovered as sister to the rest of the Egrettini. Both of these relationships received 100% bootstrap support in all five analyses (Figures 2-6).

Within the Ardeini, all analyses produced identical topologies, with most relationships receiving 100% bootstrap support. Two distinct clades within *Ardea sensu lato* were recovered: one consisting of *Ardea pacifica* as sister to *Ardea alba* and *Ardea intermedia*, and the other consisting of *Bubulcus ibis* as sister to the remaining *Ardea*. The sister relationship of *Ardea alba* and *Ardea intermedia* was modestly supported in the SVDQuartets analysis of the incomplete data set, receiving 72% bootstrap support (Figure 4).

Within the Egrettini, all analyses produced congruent topologies, with the exception of the placement of *Egretta thula*. All recovered a sister relationship between *Gorsachius leuconotus* and rest of the Egrettini and recovered *Syrigma* and *Pilherodius* as sister to each other. *Egretta* was recovered as sister to a group consisting of *Syrigma* and *Pilherodius* (Figures 2-6). However, my analyses produced contradicting topologies for *Egretta*. One recovered *Egretta thula* as sister to a group consisting of *Egretta gularis* and *Egretta garzetta*. This topology was recovered by both RAXML analyses, ASTRAL, and the analysis of the complete data set in SVDQuartets (Figures 2,3,5 & 6), was fully supported, with the exception of the SVDQuartets analysis of the complete data set, wherein it received a bootstrap support value of 82% (Figure 5). An alternative topology was recovered in the analysis of the incomplete data set in SVDQuartets, wherein *Egretta thula* was recovered as sister to *Egretta ardesiaca* and *Egretta sacra*. This relationship was poorly supported, receiving a bootstrap support value of 67% (Figure 4).

I recovered Nycticoracini *sensu* Kushlan and Hancock (2005) as non-monophyletic. *Gorsachius leuconotus* was recovered as sister to the rest of the Egrettini (Figures 2-6), with full bootstrap support. The sister relationship of *Nycticorax* and *Nyctanassa* was recovered with 100 % bootstrap support in all analyses but one. The SVDQuartets analysis of the incomplete data set recovered a bootstrap value of 82% for this relationship (Figure 4).

DISCUSSION

This study presents the most complete molecular phylogenetic hypothesis for the family Ardeidae to date. ML, quartet-based, and coalescent analyses using two genomic data sets all produced congruent and well-supported topologies for the family. Of the subfamilial

relationships, only the placement of the Tigrisomatinae in both SVDQuartets analyses was not well supported (Figures 4 & 5). In addition, I clarify the position and composition of several genera, including *Agamia*, *Ardeola*, *Pilherodius*, *Nycticorax*, *Nyctanassa* and *Ixobrychus*. My phylogenetic hypothesis lends support to a novel classification scheme (Table 4), taxonomic recommendations, and provides a topology that will be instrumental for future studies looking to evaluate the comparative biology and evolutionary history of herons.

Resolution of the backbone of the heron tree

My results suggest the following relationships among the five subfamilies *sensu* Kushlan and Hancock (2005): Tigrisomatinae is sister to all other herons, with Cochleariinae sister to a group consisting of Agamiinae, Botaurinae and Ardeinae.

The placement of Tigrisomatinae and Cochleariinae as basal lineages within the herons supports several earlier studies (Sheldon 1987a; Sheldon 1987b; Sheldon et al. 1995; McCracken and Sheldon 1997; Sheldon et al. 2000; Päckert et al. 2014) and disagrees with the findings of Huang et al. (2016), who hypothesized *Zebrilus* was sister to the rest of the herons. The recovery of Tigrisomatinae and Cochlearinae as non-sister taxa supports the findings of Sheldon et al. (1995) but disagrees with several others (Sheldon 1987a; Sheldon 1987b; McCracken and Sheldon 1997). Sheldon et al. (2000) provided equivocal support for both hypotheses. It is worthwhile to mention that the two SVDQuartets analyses only moderately supported the placement of Tigrisomatinae. This result necessitates further investigation. It has been documented that SVDQuartets has, in comparison to ML methods, has a reduced ability to infer relationships (e.g., DeGiorgio and Degnan 2010), but should approximate those obtained by ML if provided enough data (Hosner et al. 2015). If this is the case here, then the fact that my

analyses of concatenated data strongly supported the relationship of Tigrisomatinae to the rest of the herons is not surprising. Concatenated data have also been shown to be robust to large proportions of missing data (Burleigh et al. 2015).

My results also suggest a sister relationship between the Ardeinae and Botaurinae, corroborating the results of several previous studies (Sheldon 1987b; Sheldon and Kinnarney 1993; Sheldon et al. 1995; McCracken and Sheldon 1997; Sheldon et al. 2000; Chang et al. 2003; Päckert et al. 2014; Zhou et al. 2014; Zhou et al. 2016; Huang et al. 2016). The sister relationship between the Botaurinae and Ardeinae contradicts the findings of Payne and Risley (1976), who recovered the Botaurinae as sister to a group consisting of Cochleariinae, *Gorsachius*, *Nycticorax* and *Nyctanassa*.

Position and composition of genera

Position of Agamia

My results place *Agamia* as sister to the Botaurinae and Ardeinae, in disagreement with Huang et al. (2016), who recovered it as sister to *Ardeola*. I follow Kushlan and Hancock (2005) in recognizing it as a monotypic subfamily, Agamiinae.

Position and composition of Ardeola

My results suggest that *Ardeola* is sister to *Butorides*, with that clade being sister to *Ardea*, in agreement with Zhou et al. (2014). This result disagrees with the findings of Huang et al. (2016), who recovered *Butorides* as sister to *Gorsachius goisagi*, and *Ardeola bacchus* as sister to *Agamia*.

Position and composition of Gorsachius

My results suggest that *Gorsachius sensu* Gill and Donsker (2018) is polyphyletic, despite only sampling two members of this genus. The polyphyly of *Gorsachius* was originally suggested by Zhou et al. (2016), who recovered strong support for a sister relationship between *Gorsachius magnificus* and several members of *Egretta*. This result led them to suggest the resurrection of the monotypic genus *Oroanassa* for this taxon. The current study is the first to evaluate the molecular phylogenetics of *Gorsachius leuconotus*, which was recovered strong as sister to a clade consisting of *Egretta*, *Pilherodius* and *Syrigma* (Figures 2-6). *Gorsachius leuconotus* is a strictly nocturnal species (Kushlan and Hancock 2005), and its placement within Egrettini suggests that nocturnality has evolved on multiple occasions within the Ardeinae.

I recovered strong support for the sister relationship of *Gorsachius melanolophus* to the rest of the Ardeinae. This finding is contrary to the relationship recovered Zhou et al. (2016), who recovered *Gorsachius melanolophus* and *Gorsachius goisagi* as sister to *Nycticorax nycticorax*. Zhou et al. (2016) cited long-branch attraction as a potential driver of this relationship and recommended more thorough sampling before making strong conclusions regarding the phylogenetic affinities of *Gorsachius*. Although my sampling is restricted to only two members of the genus, and I am thus unable to confidently define the composition of the genus, I confidently assert that *Gorsachius* is polyphyletic, with *Gorsachius leuconotus* as sister to rest of the Egrettini, and with *Gorsachius melanolophus* as sister to the rest of the Ardeinae.

Position of Pilherodius

My results suggest that *Pilherodius* and *Syrigma* are sister taxa, with them being sister to the rest of *Egretta*. These relationships are consistent with the findings of Huang et al. (2016).

Composition of Nycticorax and Nyctanassa

My results suggest that *Nycticorax* and *Nyctanassa* are sister taxa, with them sister to the rest of Ardeini. The sister relationship of the two genera supports the results of Payne and Risley (1976) and Sheldon (1987b). Sheldon et al. (2000) and Chang et al. (2003) provided ambiguous support for both the sister relationship of *Nycticorax* and *Nyctanassa* and their unresolved placement within Ardeinae. My results contradict the findings of Zhou et al. (2014), Zhou et al. (2016), and Huang et. al (2016), who recovered *Nycticorax* as sister to *Egretta*, sister to *Gorsachius*, and sister to a group consisting of *Gorsachius* and *Butorides*, respectively.

Composition of Ixobrychus

My results suggest that *Ixobrychus*, as currently defined, is polyphyletic. I recovered strongly supported relationships that suggest that the Stripe-backed Bittern *Ixobrychus involucris* and the Least Bittern *Ixobrychus exilis* are more closely related to *Botaurus* than to other members of *Ixobrychus*. The close relationship of *Ixobrychus exilis* to *Botaurus* was first noted by Sheldon (1987b) and corroborated by Päckert et al. (2014), who recovered *Ixobrychus exilis* as sister to *Botaurus*. The sister relationship of *Ixobrychus involucris* to the group consisting of *Ixobrychus exilis* and *Botaurus* was also previously suggested by Päckert et al. (2014), although two distinct data sets produced conflicting results, with its placement as sister to other members of *Ixobrychus* also being suggested. Members of *Botaurus* are characterized by sexually

monomorphic plumage and a large size. Conversely, *Ixobrychus involucris* and *Ixobrychus exilis* are both characterized by a small size and sexually dimorphic plumage. These results suggest that sexually dimorphic plumage in herons has been lost or gained multiple times.

Composition of Egrettini

Kushlan and Hancock (2005) include *Syrigma*, *Pilherodius*, *Nyctanassa* and *Egretta* within Egrettini. I present strong support for the non-monophyly of Egrettini. As mentioned previously, *Gorsachius leuconotus* was recovered as sister to the rest of Egrettini. Additionally, *Nyctanassa* was recovered as sister to *Nycticorax*.

Composition of Nycticoracini

Kushlan and Hancock (2005) include *Nycticorax* and *Gorsachius* within Nycticoracini. Additionally, their classification of *Nycticorax* includes *Nycticorax caledonicus*, *Nycticorax nycticorax* and *Nycticorax leuconotus*. *Nyctanassa*, which has also been associated to *Nycticorax* in the past, is included within the Egrettini. I recovered non-monophyly for Nycticoracini. I recovered *Nyctanassa* as sister to *Nycticorax*, *Gorsachius leuconotus* as sister to the Egrettini and *Gorsachius melanolophus* as sister to the rest of Ardeinae. These results suggest that nocturnal behavior may have evolved or been lost multiple times within Ardeinae.

Taxonomic recommendations

Recognize Ixobrychus involucris and Ixobrychus exilis as members of the genus Botaurus

Given the strong support I recovered for the relationship of *Ixobrychus involucris* as sister to *Ixobrychus exilis* and *Botaurus*, I recommend that *Ixobrychus involucris* and *Ixobrychus exilis* be synonymized with the genus *Botaurus* Stephens, 1819, which has priority.

Recognize Dupetor flavicollis as member of the genus Ixobrychus

Given the strong support I recovered for the placement of *Dupetor flavicollis* within the genus *Ixobrychus*, I recommend that *Dupetor*, which is still recognized by some taxonomic authorities (Gill and Donsker 2018), be synonymized with the genus *Ixobrychus*, Billberg, 1828, which has priority.

Recognize Bubulcus ibis as member of the genus Ardea

Given the strong support I recovered for the placement of *Bubulcus ibis* as embedded within *Ardea*, I recommend that *Bubulcus ibis* be synonymized with the genus *Ardea*, Linnaeus, 1758, which has priority.

Recognize Goraschius leuconotus as the monotypic genus Calherodius

Given the strong support I recovered for the polyphyly of *Gorsachius*, I recommend the resurrection of the genus *Calherodius* Bonaparte, 1855, which has priority, for *Gorsachius leuconotus*.

Status of Egrettini

Given the strong support for the non-monophyly of Egrettini, I recommend subsuming it within the larger Ardeinae (Table 4). Although the Egrettini *sensu lato* may merit tribal recognition, I recommend complete taxon sampling for the subfamily before defining the composition of tribes.

Status of Nycticoracini

Given the strong support for the non-monophyly of Nycticoracini, I recommend subsuming it within the larger Ardeinae (Table 4). While the Nycticoracini *sensu lato* may merit tribal recognition, I recommend complete taxon sampling for the subfamily before defining the composition of tribes.

CONCLUSION

In summary, phylogenetic analyses ultraconserved elements resolved several long-standing questions about heron relationships. In addition to identifying the basal lineages in the family, my results also confidently resolved the composition and position of several genera that have been difficult to place: *Agamia*, *Pilherodius*, *Ardeola, Nycticorax, Nyctanassa,* and *Ixobrychus*. Lastly, my results recover non-monophyly for several traditionally recognized genera and tribes, including: *Ardea*, *Ixobrychus*, *Gorsachius,* Nycticoracini and Egrettini. The phylogenetic hypothesis presented here will be of use to studies seeking to evaluate the evolutionary history of herons.

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TABLES AND FIGURES

Table 1. Current classification of herons.

Family Ardeidae
- Subfamily Ardeinae
  - Tribe Ardeini
    - *Ardea*, *Butorides*, *Ardeola*
  - Tribe Egrettini
    - *Egretta, Syrigma, Pilherodius, Nyctanassa*
  - Tribe Nycticoracini
    - *Nycticorax*, *Gorsachius*
- Subfamily Botaurinae
  - *Botaurus*, *Ixobrychus*, *Zebrilus*
- Subfamily Tigrisomatinae
  - *Tigrisoma*, *Zonerodius*, *Tigriornis*
- Subfamily Agamiinae
  - *Agamia*
- Subfamily Cochleariinae
  - *Cochlearius*

Table 2. Synonymies of genera used in this chapter.

Genus	Bock 1956	Payne 1979	Kushlan and Hancock 2005	Gill and Donsker 2018
Agamia	*Agamia*	*Agamia*	*Agamia*	*Agamia*
Ardea	*Ardea*	*Ardea*	*Ardea*	*Ardea*
Ardeola	*Ardeola*	*Ardeola*	*Ardeola*	*Ardeola*
Botaurus	*Botaurus*	*Botaurus*	*Botaurus*	*Botaurus*
Bubulcus	*Ardeola*	*Egretta*	*Ardea*	*Bubulcus*
Butorides	*Butorides*	*Ardeola*	*Butorides*	*Butorides*
Calherodius	*Gorsachius*	*Nycticorax*	*Nycticorax*	*Gorsachius*
Cochlearius	*Cochlearius*	*Cochlearius*	*Cochlearius*	*Cochlearius*
Dupetor	*Ixobrychus*	*Ixobrychus*	*Ixobrychus*	*Dupetor*
Egretta	*Egretta*	*Egretta*	*Egretta*	*Egretta*
Gorsachius	*Gorsachius*	*Nycticorax*	*Gorsachius*	*Gorsachius*
Ixobrychus	*Ixobrychus*	*Ixobrychus*	*Ixobrychus*	*Ixobrychus*
Nyctanassa	*Nycticorax*	*Nyctanassa*	*Nyctanassa*	*Nyctanassa*
Nycticorax	*Nycticorax*	*Nycticorax*	*Nycticorax*	*Nycticorax*
Oroanassa	*Gorsachius*	*Nycticorax*	*Gorsachius*	*Gorsachius*
Pilherodius	*Pilherodius*	*Pilherodius*	*Pilherodius*	*Pilherodius*
Syrigma	*Nycticorax*	*Syrigma*	*Syrigma*	*Syrigma*
Tigriornis	*Tigriornis*	*Tigriornis*	*Tigriornis*	*Tigriornis*
Tigrisoma	*Tigrisoma*	*Tigrisoma*	*Tigrisoma*	*Tigrisoma*
Zebrilus	*Zebrilus*	*Zebrilus*	*Zebrilus*	*Zebrilus*
Zonerodius	*Zonerodius*	*Zonerodius*	*Zonerodius*	*Zonerodius*

Table 3. Samples used in this study, including locality information and coverage statistics.

Taxon	Sample	Country	Number of UCEs	Average UCE contig length	Average coverage of UCE contigs
Agamia agami	LSUMNS B12815	Bolivia	4711	764.1	14.5
Ardea alba	LSUMNS B1343	USA	4308	1164.9	70.7
Ardea cinerea	**KUNHM 21788**	Spain	4735	1131.2	87
Ardea goliath	LSUMNS B10361	Zoo/captive	4075	1166	37.1
Ardea intermedia	MSB 177136	South Africa	4716	1093.7	47.9
Ardea melanocephala	LSUMNS B39300	Ghana	4682	1025.5	44.2
Ardea pacifica	UWBM 62925	Australia	4710	1155.7	111.2
Ardea purpurea	LSUMNS B39468	Ghana	4576	1165.9	93.2
Ardeola bacchus	UAM 26000	USA	4678	1076.7	78.3
Ardeola ralloides	LSUMNS B34283	South Africa	4521	1031.3	63.5
Ardeola speciosa	AMNH DOT17256	Singapore	4476	1076.1	37.4
Balaeniceps rex	LSUMNS B19208	Zoo/captive	4579	1338.4	90
Botaurus lentiginosus	LSUMNS B18981	USA	4672	1006.5	67.9
Botaurus poiciloptilus	UWBM 80401	Zoo/captive	4586	997	56.3
Bubulcus ibis	LSUMNS B19756	USA	4109	1131.2	53
Butorides striata	LSUMNS B12810	Bolivia	4360	1095.6	112.3
Butorides virescens	KUNHM 9507	El Salvador	4663	1148.3	132
Cochlearius cochlearius	LSUMNS B1339	Zoo/captive	4672	984.2	58.1
Dupetor flavicollis	UWBM 67898	Solomon Islands	4706	1100.8	64.1
Egretta ardesiaca	SDMNH 51906	Zoo/captive	4711	1140.3	26
Egretta caerulea	LSUMNS B5283	USA	4640	1019.5	48.3
Egretta garzetta	LSUMNS B62605	Kuwait	4332	1112.5	105.4
Egretta gularis	LSUMNS B62603	Kuwait	4403	1120	97.7
Egretta rufescens	LSUMNS B6449	USA	4752	1236.6	74
Egretta sacra	UAM	Australia	4700	1255.8	37.5

	17951				
Egretta thula	LSUMNS B6385	USA	4692	1086.9	75.7
Egretta tricolor	LSUMNS B19408	USA	4636	1176	127.9
Gorsachius leuconotus	LSUMNS B45084	Ghana	4523	1174.9	74.5
Gorsachius melanolophus	KUNHM 10441	China	4640	1085.2	28.5
Ixobrychus cinnamomeus	AMNH DOT17237	Singapore	4761	1068.5	126.8
Ixobrychus eruhythmus	AMNH DOT17239	Singapore	4687	1040.9	1.3
Ixobrychus exilis	LSUMNS B3882	USA	4659	1254	81.9
Ixobrychus involucris	LSUMNS B35927	Trinidad and Tobago	4639	1133	50.9
Ixobrychus dubius	AMNH DOT17848	Australia	4679	1040.4	41.6
Ixobrychus sinensis	FLMNH 44361	USA	4728	1141.1	162.6
Ixobrychus sturmii	UWBM 104503	Malawi	4549	1094.4	52.4
Nyctanassa violacea	LSUMNS B15549	United States	3538	1136.5	58.5
Nycticorax caledonicus	KUNHM 10686	Australia	4715	1231.7	181
Nycticorax nycticorax	CHU006	Mozambique	4502	1185.5	70.1
Pelecanus occidentalis	LSUMNS B36186	United States	4678	1309.3	97.4
Pilherodius pileatus	KUNHM 1247	Guyana	4475	1183.5	84.2
Plegadis falcinellus	LSUMNS B41209	United States	4717	1162.5	106.4
Syrigma sibilatrix	LSUMNS B6613	Bolivia	4708	1182.5	46.1
Tigriornis leucolopha	UMMZ 235185	Gambia	4705	1175.6	74.8
Tigrisoma fasciatum	LSUMNS B44561	Peru	4574	1116.9	48.2
Tigrisoma lineatum	**KUNHM 3145**	Paraguay	4708	1138.6	52.4
Tigrisoma mexicanum	LSUMNS B46531	Panama	4685	1082.6	58.1
Zebrilus undulatus	LSUMNS B12873	Bolivia	4642	1130.9	69.1
		Average	4581.5	1122.3	72.5

Table 4. Revised classification of Ardeidae.

Family Ardeidae

Subfamily Ardeinae

Ardea, *Butorides*, *Ardeola*
Egretta, Syrigma, Pilherodius,
Calherodius, Nyctanassa,
Nycticorax, *Gorsachius*

Subfamily Botaurinae

Botaurus, *Ixobrychus*, *Zebrilus*

Subfamily Tigrisomatinae

Tigrisoma, *Zonerodius*, *Tigriornis*

Subfamily Agamiinae

Agamia

Subfamily Cochleariinae

Cochlearius

Figure 1. Phylogenetic hypothesis proposed by Sheldon (1987b).

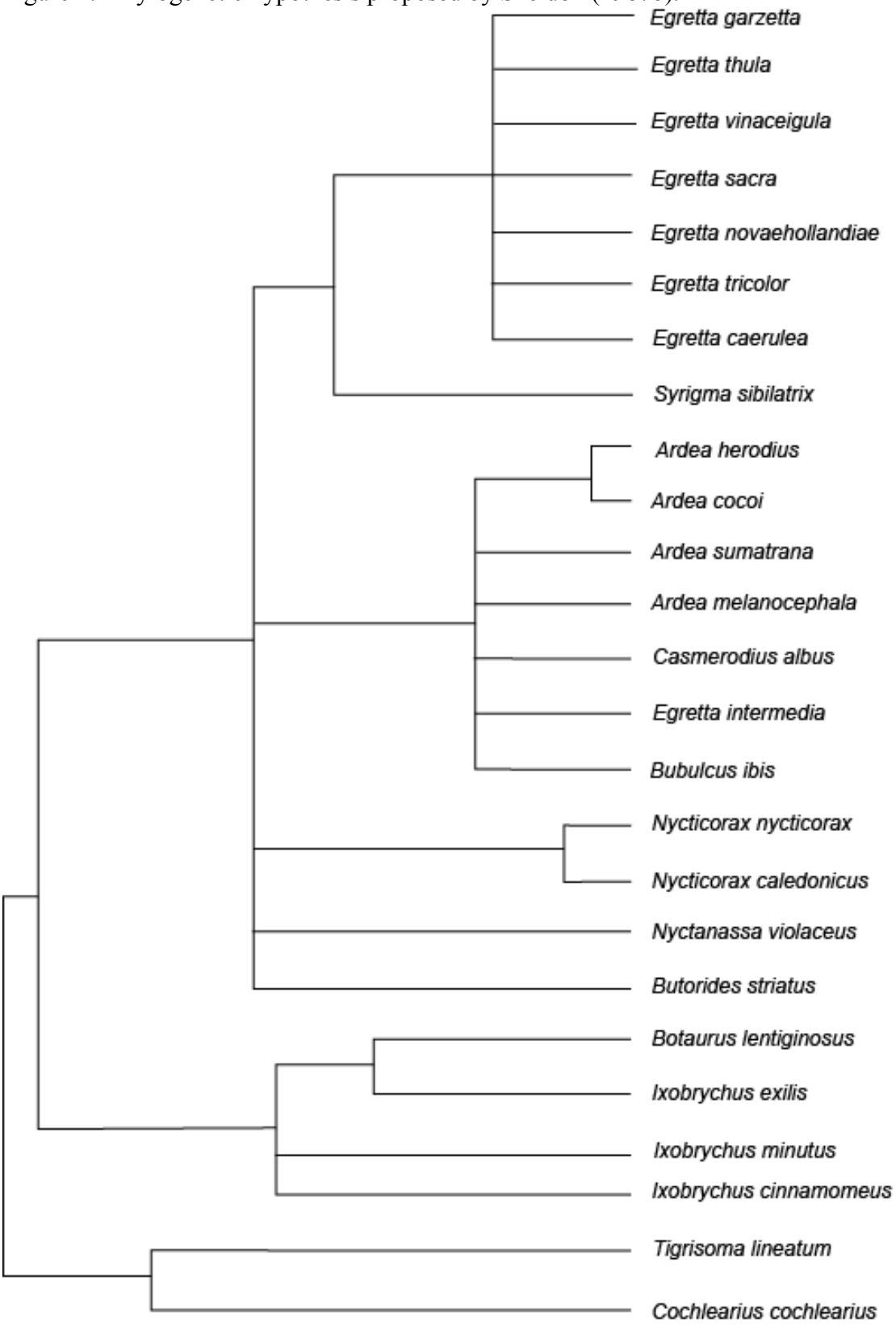


Figure 2. RAxML tree of the complete data set.

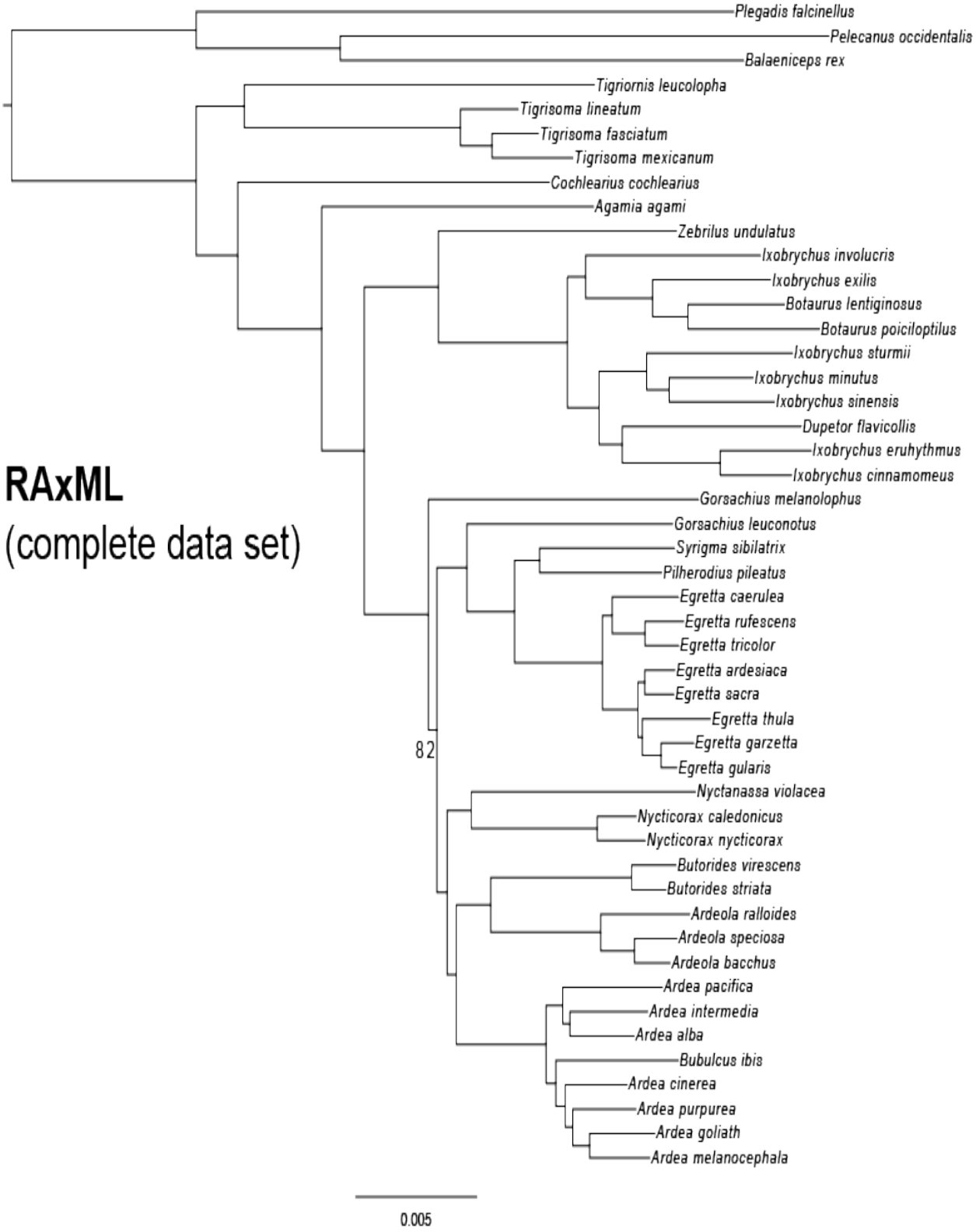


Figure 3. RAxML tree of the incomplete data set.

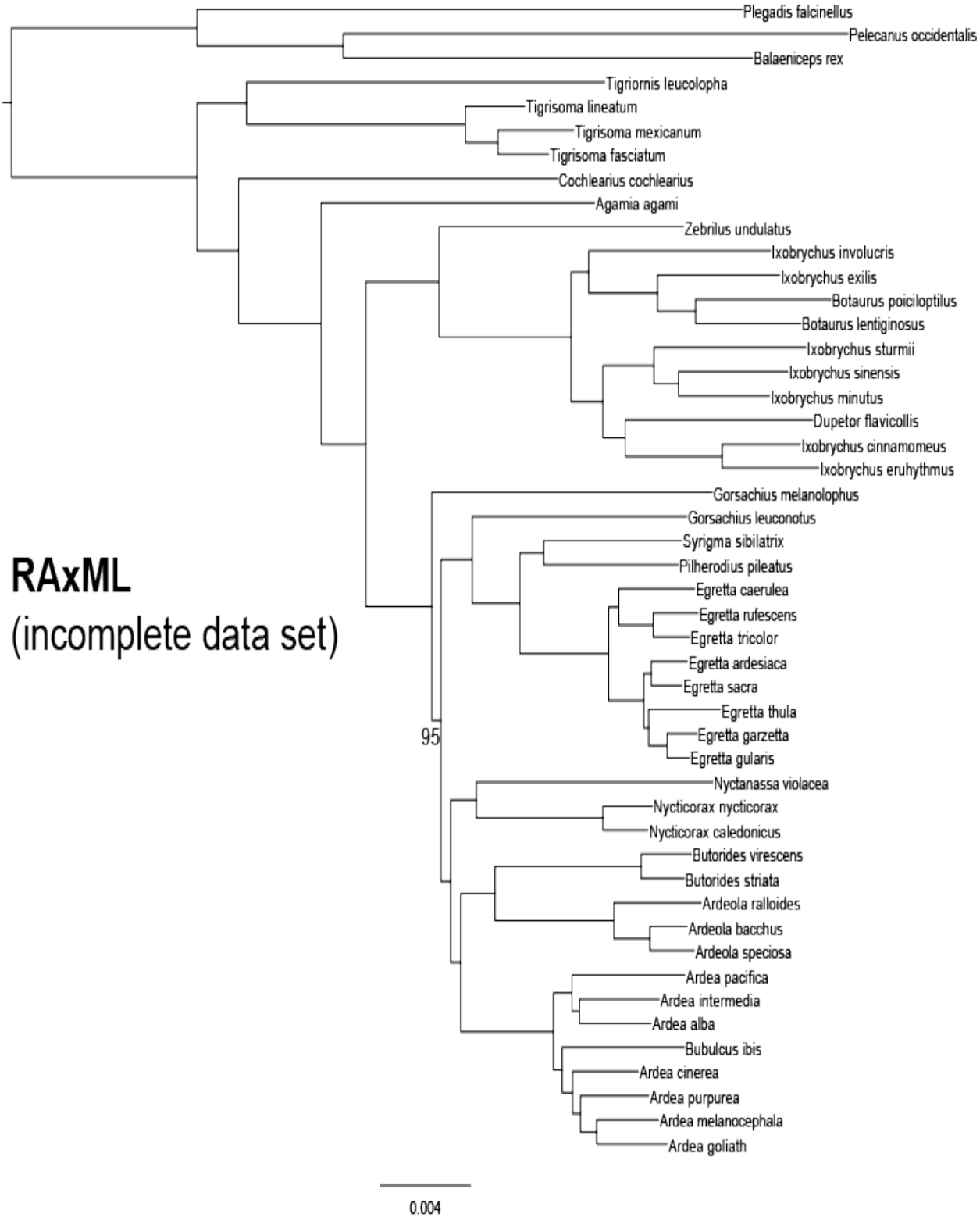


Figure 4. SVDQuartets tree of the incomplete data set.

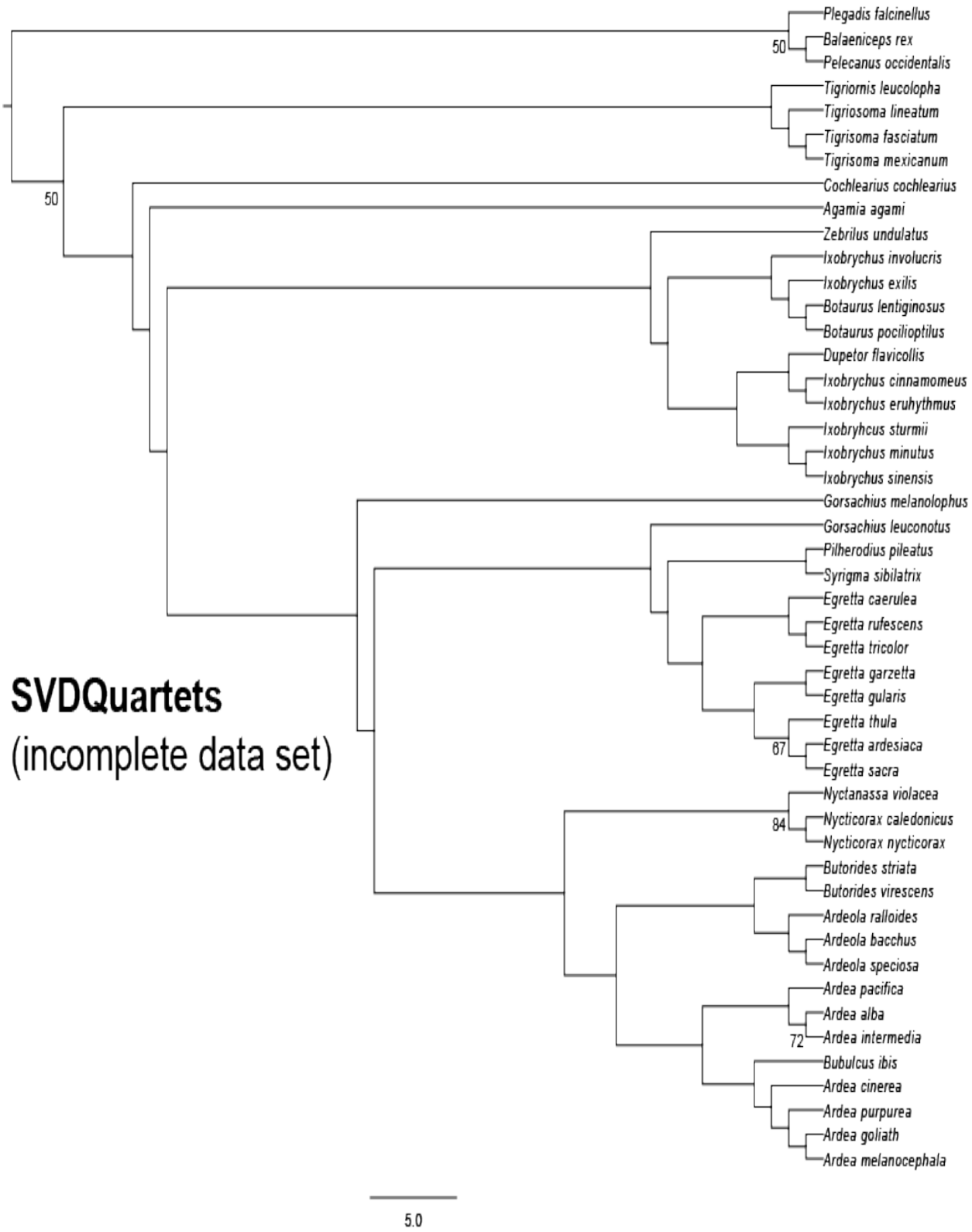


Figure 5. SVDQuartets tree of the complete data set.

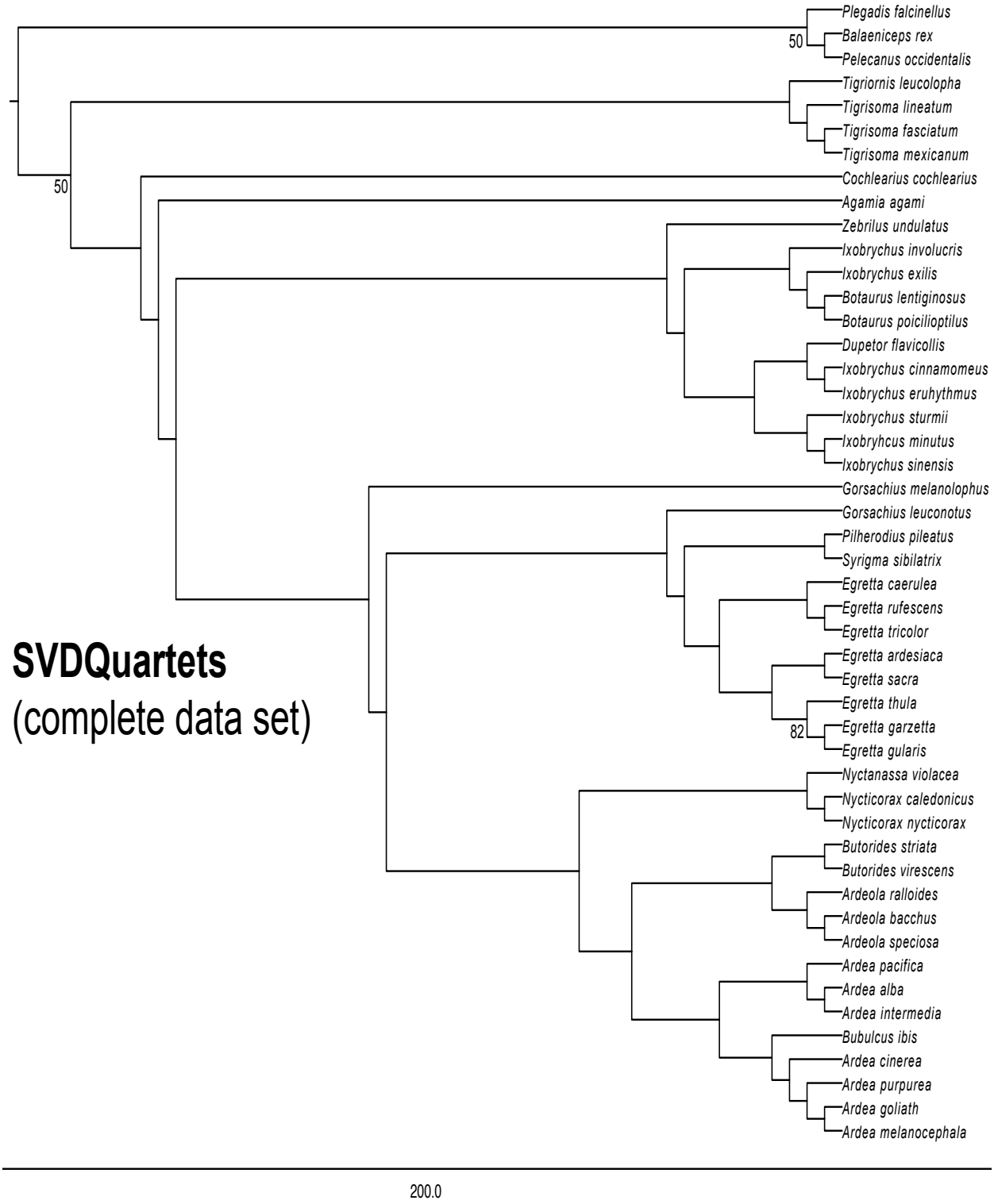
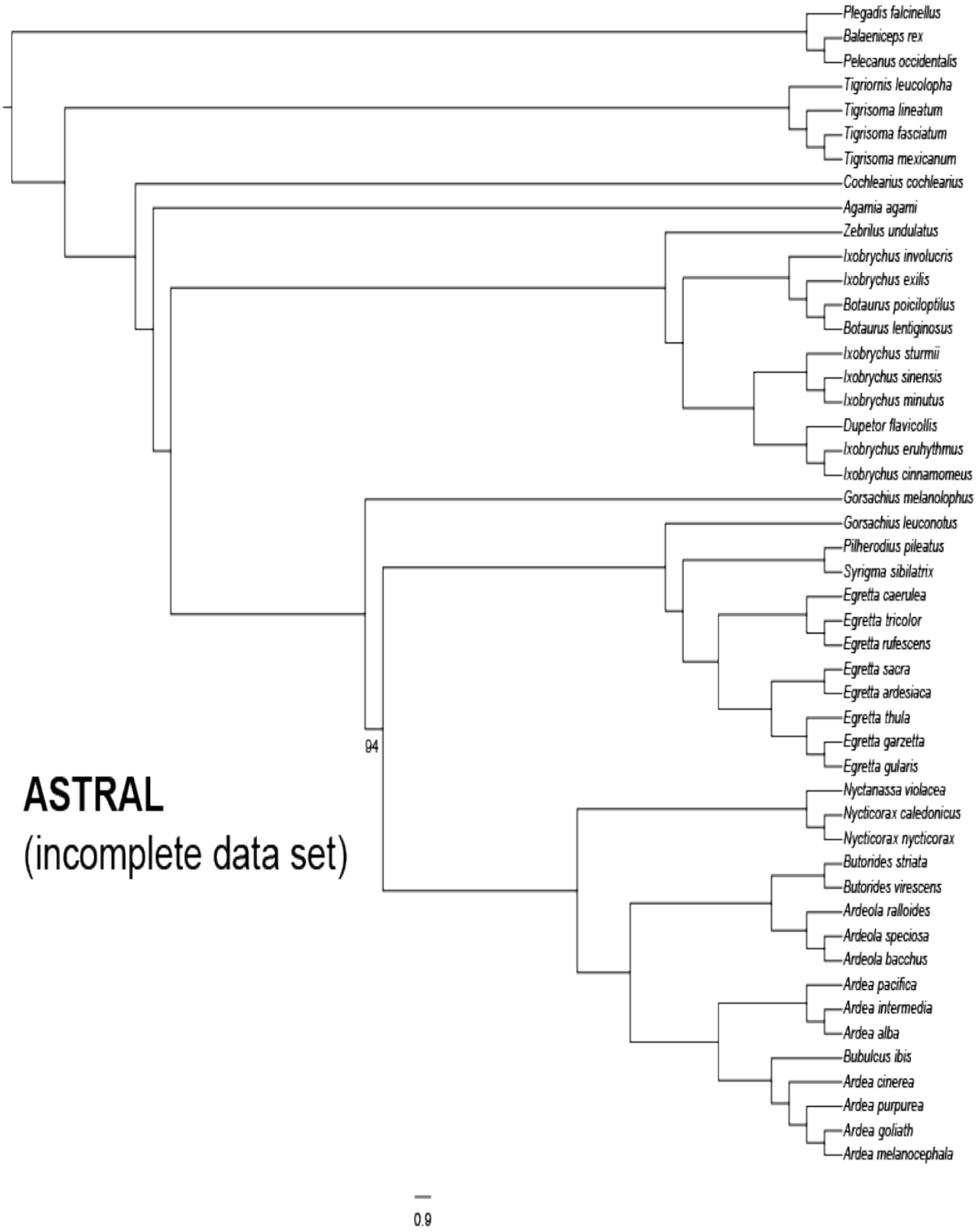


Figure 6. ASTRAL tree of the incomplete data set.


Plegadis falcinellus
Balaeniceps rex
Pelecanus occidentalis
Tigriornis leucolopha
Tigrisoma lineatum
Tigrisoma fasciatum
Tigrisoma mexicanum
Cochlearius cochlearius
Agamia agami
Zebrilus undulatus
Ixobrychus involucris
Ixobrychus exilis
Botaurus poiciloptilus
Botaurus lentiginosus
Ixobrychus sturmii
Ixobrychus sinensis
Ixobrychus minutus
Dupetor flavicollis
Ixobrychus eruhythmus
Ixobrychus cinnamomeus
Gorsachius melanolophus
Gorsachius leuconotus
Pilherodius pileatus
Syrigma sibilatrix
Egretta caerulea
Egretta tricolor
Egretta rufescens
Egretta sacra
Egretta ardesiaca
Egretta thula
Egretta garzetta
Egretta gularis
Nyctanassa violacea
Nycticorax caledonicus
Nycticorax nycticorax
Butorides striata
Butorides virescens
Ardeola ralloides
Ardeola speciosa
Ardeola bacchus
Ardea pacifica
Ardea intermedia
Ardea alba
Bubulcus ibis
Ardea cinerea
Ardea purpurea
Ardea goliath
Ardea melanocephala
94
ASTRAL
(incomplete data set)
0.9

FIGURES AND TABLE CAPTIONS

Table 1. Classification of the herons *sensu* Kushlan and Hancock (2005).

Table 2. Synonymies of genera used in chapter, proposed by the most recent classifications for the herons.

Table 3. Samples used in this study, including locality information and coverage statistics. Taxonomy follows Gill and Donsker 2018. Institutional abbreviations are as follows: Louisiana State University Museum of Natural Science (LSUMNS), University of Kansas Natural History Museum (KUNHM), American Museum of Natural History (AMNH), Field Museum of Natural History (FMNH), University of Alaska Museum (UAM), University of Washington Burke Museum (UWBM), Florida Museum of Natural History (FLMNH), Museum of Southwestern Biology (MSB), San Diego Natural History Museum (SDNHM), University of Michigan Museum of Zoology (UMMZ). Samples in bold were extracted using a Maxwell RSC Blood DNA kit.

Table 4. Revised classification of Ardeidae, informed by results of this study.

Figure 1. Phylogenetic hypothesis proposed by Sheldon (1987b) based on DNA-DNA hybridization data.

Figure 2. Maximum likelihood (ML) tree of the complete and concatenated data set. Tree was estimated in RAxML assuming a general time reversible model of rate substitution and gamma-distributed rates among sites. Bootstrap support assessed using 1000 rapid replicates. Bootstrap support 100% unless otherwise noted.

Figure 3. Maximum likelihood (ML) tree of the incomplete and concatenated data set. Tree was estimated in RAxML assuming a general time reversible model of rate substitution and gamma-distributed rates among sites. Bootstrap support assessed using 1000 rapid replicates. Bootstrap support 100% unless otherwise noted.

Figure 4. Quartet tree of the incomplete and concatenated data set. Tree was estimated in SVDQuartets. Bootstrap support assessed using 100 replicates. Bootstrap support 100% unless otherwise noted.

Figure 5. Quartet tree of the complete and concatenated data set. Tree was estimated in SVDQuartets. Bootstrap support assessed using 100 replicates. Bootstrap support 100% unless otherwise noted.

Figure 6. Summary statistic species tree of the incomplete data set. Tree was estimated in ASTRAL, using gene trees estimated in RAxML as input. Bootstrap support assessed using 100 multilocus replicates, resampling both loci and sites within loci. Bootstrap support 100% unless otherwise noted.