INTEGRATING PHYLOGENETIC RELATIONSHIPS AND POPULATION STRUCTURE FOR CONSERVATION PLANNING IN GALLIFORMES

by

SOO HYUNG EO

(Under the Direction of John P. Carroll)

ABSTRACT

Genetic components and evolutionary processes are critical to explain variation in both extinction risks among species and population structures within species because they generate and maintain adaptive biological variation.

To understand phylogenetic relationships among fowl species, I constructed supertree of orders Galliformes (chicken-like birds) and Anseriformes (duck-like birds). Using formal algorithmic procedures and source trees available, supertree methods are able to represent such a large clade phylogeny, which is almost impossible with conventional approaches using either molecular or non-molecular data. My Galloanserae supertree represents one of the most comprehensive estimates for the group to date, including 376 species (83.2% of all species; all 162 Anseriformes and 214 Galliformes). The use of this phylogenetic supertree enables us to apply comparative analysis, considering phylogenetic independence, to describing their remarkable diversity of life history, morphology, behavioral ecology, conservation biology, and other evolutionary processes across species.

Below species level, it is critical that we understand genetic identity as the basis for the conservation and management of the species and surrounding habitat. Using mitochondrial and

nuclear microsatellite loci, I investigated intraspecific genetic relationships among northern bobwhites (*Colinus virginianus*). There was extremely high genetic differentiation between isolated Arizona northern bobwhites (masked bobwhite, *C. v. ridgwayi*) and the other subspecies (*C. v. marilandicus*, *C. v. virginianus*, *C. v. floridanus*, *C. v. mexicanus*, *C. v. taylori*, and *C. v. texanus*). Based on genetic structure and geographic ranges, my results suggest that each of *C. v. ridgwayi* and *C. v. floridanus* should be considered as a distinct unit for conservation or management, supporting current subspecies limits. However, *C. v. virginianus*, *C. v. marilandicus*, *C. v. mexicanus,* and *C. v. taylori* may be considered a single management unit because levels of genetic divergence among these putative subspecies were quite low. Among all analyzed subspecies, masked bobwhite has the lowest diversity in all genetic information. Therefore, it is highly recommended to set conservation priority to the masked bobwhites as an independent conservation unit.

INDEX WORDS: Supertree, Galloanserae, Anseriformes, Galliformes, *Colinus virginianus*, Population structure, Units for Conservation and management

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SOO HYUNG EO

B.S., The Seoul National University, South Korea, 1999

M.S., The Seoul National University, South Korea, 2001

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by

SOO HYUNG EO

Major Professor: John P. Carroll

Committee: Michael J. Conroy
Daniel E. L. Promislow
John P. Wares

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Global biodiversity currently faces an extinction crisis at an alarming rate that is unprecedented (Novacek and Cleland 2001; Pimm et al. 1995). About 20% of mammalian and 12% of avian species are listed by IUCN as threatened with extinction (IUCN 2007). At the population level, it is estimated that annually 1% of habitats and populations are currently losing (Balmford et al. 2003; Hughes et al. 1997). However, not all populations, species, or lineages are equally likely to be threatened and to become extinct. Some species are much more likely to become extinct than others. For example, avian families such as parrots (Psittacidae), albatrosses (Procellaridae), and pheasants (Phasianidae) have significantly more threatened species than expected by chance, whereas families such as woodpeckers (Picidae) and cuckoos (Cuculidae) have significantly fewer threatened species (Bennett and Owens 1997; IUCN 2007). This suggests that threatened taxa tend to be more closely related to one another in their clades and that loss of evolutionary diversity tends to be made worse in those clades (Purvis et al. 2000a). Even within species, declining populations have been more often found at range margins and in fragmented patches than within central populations with continuous ranges, thereby also exhibiting low genetic diversity and greater genetic differentiation (Channell and Lomolino 2000; Eckert et al. 2008). In this context, many researchers have argued that vulnerability to extinction of species or local populations may be commonly associated with not only human-induced direct threats to biodiversity, but also demographic or ecological aspects, such as small population size and high

habitat specificity (e.g., Johnson et al. 2002; MacArthur and Wilson 1967; Owens and Bennett 2000; Purvis et al. 2000b), and evolutionarily adaptive or genetic traits such as large body size, slow reproductive rates and low genetic variability that make species susceptible to extinction (e.g., Bennett and Owens 1997; Frankham et al. 2002; Keane et al. 2005; Long et al. 2007; Price and Gittleman 2007).

Genetic components and evolutionary processes are critical to explain variation in both extinction risks among species and population structures within species because they generate and maintain adaptive biological variation (Avise 2000; Futuyma 2005; Mace and Purvis 2008; Norris and Pain 2002). It is important to retain the ability for taxa to adapt to new environments in a rapidly changing world (Mace and Purvis 2008). Consequently, efficient and systematic conservation efforts have to incorporate both ecological and evolutionary processes (Rouget et al. 2006). After recognizing the importance of genetic components and evolutionary processes, as well as ecological importance, the next practical step in conservation biology is delineating 'units' of biodiversity (Moritz 1994; Ryder 1986). Traditionally, species is the basic unit of interest in conservation biology (Agapow et al. 2004; Barraclough and Nee 2001), therefore delineating and identifying species is important to design species-specific conservation and management. In this respect, a well-resolved phylogenetic structure can provide a basis when we select specific species as units for conservation action. However, the scale of conservation efforts may vary; conservation biologists not only target a specific species, but also restore important habitats and populations at local scales and protect widespread species, genera, and families at global scales (Norris and Pain 2002). Given that we do not have time, man-power, and finances enough to undertake conservation efforts for all taxa, setting conservation priorities and correctly allocating limited conservation resources should be based on comparison of the

relative importance of all possible conservation units (Fisher and Owens 2004; Purvis et al. 2000c). In my dissertation, I describe phylogenetic relationships among species and intraspecific population structures for conservation planning in Galliformes, integrating macro- and micro-level molecular ecology, evolutionary genetics, and applications to the conservation biology of this avian group.

In the first part (Chapters 2) of my dissertation, focusing on macro-level evolution and conservation biology, I construct phylogenetic supertree of orders Galliformes (chicken-like birds) and Anseriformes (duck-like birds). Based on this phylogenetic relationship, we may illustrate life history, ecological, and anthropogenic correlates of extinction risk in Galliformes. The Galloanserae (Galliformes and Anseriformes), comprising about 450 species with remarkable diversity of their life history traits, presents an exceptional group for studying a wide range of ecology, evolutionary biology, and conservation biology (del Hoyo et al. 1992, 1994; Dickinson 2003). Although much phylogenetic information has been presented in the Galloanserae (e.g., Armstrong et al. 2001; Birks and Edwards 2002; Crowe et al. 1992; Dimcheff et al. 2002; Donne-Gousse et al. 2002; Drovetski 2002; Livezey 1986, 1991, 1996; Pereira et al. 2002), thus far genus-level relationships have been functioned as the most detailed phylogenies for this group (e.g., Crowe et al. 2006; Livezey 1997). Using formal algorithmic procedures and source trees available, supertree methods are able to represent such a large clade phylogeny, which is almost impossible with conventional approaches using either molecular or non-molecular data (Bininda-Emonds et al. 2004; Sanderson et al. 2003). In Chapter 2, I construct species-level phylogenetic relationships of the Galloanserae using the supertree approach.

Comparative methods are frequently applied to testing hypotheses on adaptations and other evolutionary phenomena (Futuyma 2005). However, it is not appropriate to treat values of

traits from closely related species as independent because such traits are often shared through common descent rather than independent evolution (Felsenstein 1985; Harvey and Pagel 1991). The use of Galliformes phylogenetic supertrees enables us to apply comparative analysis, considering phylogenetic independence, to describing their remarkable diversity of life history, morphology, behavioral ecology, and other evolutionary processes across species.

At the micro-level of molecular ecology, the second part (Chapters 3 and 4) of my dissertation addresses subspecies-level phylogenetic relationships and patterns of genetic population structure in a species, Northern Bobwhite *Colinus virginianus*, using mitochondrial and nuclear genome analyses. The species, widely distributed in the North America, is currently undergoing population declines throughout most of its native geographic range (Brennan 1999; Burger 2002; Carroll 1994; Johnsgard 1988). However, we lack basic information on their intraspecific systematics and genetic structure of populations. Although most conservation biologists believe that genetically differentiated populations within species (such as evolutionarily significant units or management units) require separate genetic management (Crandall et al. 2000; Moritz 1994; Palsboll et al. 2007), many introductions and translocations of northern bobwhites have been undertaken, without genetic consideration, for the purpose of recovery from population decline (see references in Scott 1985). It is critical that we understand their genetic identity as the basis for the conservation and management of the species and surrounding habitat. In Chapter 3, I investigate intraspecific genetic relationships among four putative northern bobwhite subspecies (*C, v. marilandicus*, *C. v. virginianus*, *C. v. mexicanus*, and *C. v. floridanus*) in the eastern United States, using mitochondrial DNA control region sequences. Also, I examine if populations have experienced recent demographic expansion and colonization processes.

Despite its relative abundance and widespread distribution of northern bobwhites, northern bobwhite has been listed as "Near Threatened" by the IUCN since 2001 (IUCN 2007). Particularly, one subspecies (masked bobwhite *C. v. ridgwayi*) is listed under the Endangered Species Act of 1973 (USFWS 1995). In fact, this subspecies is geographically isolated and restricted to only one reintroduced population in Arizona, with population of only 1000 – 2000 individuals (Carroll 1994; Hernandez et al. 2006; Kuvlesky et al. 2000). However, we have little information on if northern bobwhites including this endangered subspecies have high levels of genetic diversity, are connected with adjacent or related populations, or are genetically managed as some separate units for conservation. In Chapter 4, I employ both mitochondrial and nuclear microsatellite loci to examine patterns and levels of genetic differentiation for northern bobwhite populations and to describe possible factors responsible for shaping variable genetic structures across widespread or isolated populations in the species.

Finally, in Chapter 5, I summarize integrative conclusion that can not only contribute to pure natural science itself, but also act as a powerful tool for directing effective conservation planning and management implications for this avian group.

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CHAPTER 2

A PHYLOGENETIC SUPERTREE OF THE FOWL (GALLOANSERAE, AVES)[1]

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ABSTRACT

The fowls (Anseriformes and Galliformes) comprise one of the major lineages of birds and occupy almost all biogeographic regions of the world. The group contains the most economically important of all bird species, each with a long history of domestication, and is an ideal model for studying ecological and evolutionary patterns. Yet, despite both the socio-economic and biological importance of fowls, the species-level relationships within this clade remain controversial. Here we used the supertree method matrix representation with parsimony to generate a robust estimate of species-level relationships of fowls. The supertree represents one of the most comprehensive estimates for the group to date, including 376 species (83.2% of all species; all 162 Anseriformes and 214 Galliformes) and all but one genera. The supertree was well-resolved (81.1%) and supported the monophyly of both Anseriformes and Galliformes. The supertree supported the partitioning of Anseriformes into the three traditional families Anhimidae, Anseranatidae, and Anatidae, although it provided relatively poor resolution within Anatidae. For Galliformes, the majority-rule supertree was largely consistent with the hypothesis of sequential sister-group relationships between Megapodiidae, Cracidae, and the remaining Galliformes. However, our species-level supertree indicated that more than 30% of the polytypic genera examined were not monophyletic, suggesting that results from genus-level comparative studies using the average of the constituent species' traits should be interpreted with caution until analogous species-level comparative studies are available. Areas where the supertree was either poorly resolved or incomplete reflected gaps or conflict within the existing phylogenetic database, highlighting areas in need of more study. Even so, our supertree will provide a valuable foundation for understanding the diverse biology of fowls in a robust phylogenetic framework.

INTRODUCTION

The fowls (Galloanserae; ducks, chicken, and allies) are generally regarded as a monophyletic group (but see Olson & Fecuccia 1980; Ericson 1996, 1997) that, according to Dickinson (2003), consist of eight families with 452 species. Fowls, which are typically separated into duck-like (Anseriformes) and chicken-like species (Galliformes), include the most economically important birds on earth. Many species in this group have a long history of domestication for socio-economic reasons (e.g. food, game, feather, or display, among others), including chicken (e.g. *Gallus gallus*), quails (e.g. *Coturnix japonica* and *Colinus virginianus*), ring-necked pheasants (*Phasianus colchicus*), turkeys (e.g. *Meleagris gallopavo*), guinea fowls (e.g. *Numida meleagris*), peafowls (*Pavo cristatus*), ducks (e.g. *Anas platyrhynchos*), and geese (e.g. *Anser anser* and *Anser cygnoides*). The global economic value of domesticated fowls is enormous. For example, more domestic chicken meat (over 68 million tons) than beef was produced worldwide in 2004 (FAO 2007). Income from eggs and poultry in the United States was approximately US$29 billion in 2004 (USDA 2007). Hunting of migratory birds (e.g. ducks and geese) in the United States generates US$1.3 billion annually for thousands of small businesses (USFWS 2007), and game shooting in the United Kingdom similarly supports some 70,000 full-time jobs (PACEC 2006).

Fowls are likewise of particular interest to many biologists. The group comprises the sister group of all remaining species of Neognathae [all living birds with the exception of tinamous (Tinamidae) and ratites (Struthionidae, Rheidae, Casuariidae, Dromaiidae, and Aterygidae)], and occupies almost all major biogeographic regions of the world (Cracraft *et al* 2004). Despite this deep divergence and worldwide distribution, Anseriformes and Galliformes together possess extremely restricted extant species richness relative to their sister group

(Neoaves), which covers over 9,000 species (Dickinson 2003). Even so, fowls display a remarkable life-history and behavioral diversity as well as morphological plasticity (del Hoyo *et al* 1992; Dunning 1993; del Hoyo *et al* 1994; Kear 2005). For example, species within Galliformes show more than a 100-fold difference in body mass (e.g. from <100g for *Coturnix japonica* to ~10,000g for *Meleagris gallopavo*), and more than a 20-fold difference in clutch size (e.g. from one for *Lophura bulweri* to >20 for *Alectura lathami*). Many galliform species tend to be sedentary, whereas most anseriform species migrate long distances. Within Galliformes, some grouse are characterized by adaptations to open habitats, whereas megapodes and cracids are adapted to forest habitats. Anseriformes are adapted generally to an aquatic lifestyle (e.g. webbed feet), but their reliance on the aquatic habitat differs widely among species. Swans and geese often feed on land at some distance from water, whereas most ducks forage in or close to water. Some fowl species (e.g. *Crax alberti* and *Anas laysanensis*) are recognized as being critically endangered (IUCN 2007), whereas others (e.g. *Phasianus colchicus* and *Anas platyrhynchos*) are exploited as overabundant game species. Such remarkable diversity in Galloanserae makes it an exceptional group for studying a wide range of questions in ecology, evolution, conservation, and management.

Biologists often employ a comparative approach to recognize, test, and interpret adaptive patterns and processes in ecology and evolution. To do so properly, a phylogenetic framework is essential to account for the nonindependence among taxa that arises through the process of descent with modification (Felsenstein 1985b; Harvey & Pagel 1991). Thus, a large, well-resolved (species-level) phylogeny, in addition to its systematic value, represents an indispensable tool for testing broad-scale hypotheses in nature, greatly increasing the statistical power of the associated comparative analyses. Currently, however, it is generally not possible to

build large, comprehensive trees from a direct, conventional analysis of true biological characters, such as DNA sequences, due to uneven distribution of research effort across taxa resulting in insufficient homologous data (Sanderson *et al* 2003; Bininda-Emonds 2005). This state of affairs also holds for Galloanserae, with a general lack of large species-level trees from any single molecular, morphological, or combined dataset. To date, the most comprehensive trees for each of Anseriformes and Galliformes are genus-level trees, with Livezey (1997) summarizing the findings of several partial phylogenies for Anseriformes based on morphology and Crowe *et al* (2006) deriving a tree for Galliformes from an analysis of morphological and molecular data from 158 out of the 292 extant species.

Instead, supertree analysis provides an alternative method to generate comprehensive and rigorous estimates of phylogeny (Sanderson *et al* 1998; Bininda-Emonds, Gittleman *et al* 2004). Using formal algorithmic procedures, this method combines multiple existing and overlapping source trees, each ideally based on independent data sets (see Gatesy *et al* 2002), and therefore is able to use more of the information present in the global systematic database. Despite repeated criticism that supertree approaches use only the topological information of the source trees and thus lose contact with the raw data (e.g. Springer & de Jong 2001; Gatesy *et al* 2002), simulation studies have repeatedly shown that supertrees built with sufficiently large and numerous source trees represent the phylogenetic information provided by the source trees accurately (Bininda-Emonds & Sanderson 2001; Chen *et al* 2003). With these advantages, comprehensive supertrees have been built for a wide range of animals and plants, including all extant mammal species (Bininda-Emonds *et al* 2007), seabirds (Kennedy & Page 2002), shorebirds (Thomas *et al* 2004), oscine passerine birds (Jønsson & Fjeldså 2006), dinosaurs (Pisani *et al* 2002), grasses (Salamin *et al* 2002), and angiosperms (Davies *et al* 2004).

Here, we use the supertree method of matrix representation with parsimony (MRP: Baum 1992; Ragan 1992) to generate a robust estimate of species-level phylogenetic relationships within Galloanserae. The major objectives of this study are: 1) to provide a comprehensive, global view of the group's phylogenetic relationships; 2) to compare this topology to other comprehensive fowl phylogenies based on the conventional analysis of molecular or morphological characters (e.g. Livezey 1997; Crowe *et al* 2006); and 3) to provide a phylogenetic framework for future comparative studies of fowl ecology, evolution, conservation and management.

MATERIALS AND METHODS

Source Tree Collection

Phylogenetic information for Galloanserae was collated from the published literature by searching online databases, the Web of Science and Zoological Record for the years 1971 - 2006. We used the following search terms: phylogen*, phenogram*, cladogram*, cladistic*, taxonom*, or fossil* (where the asterisks represent wildcards) in combination with any scientific name of each fowl order, family, subfamily, or genus (as given in Dickinson 2003) or any major fowl common name (e.g. fowl, gamebird, grouse, quail, pheasant, waterfowl, duck, goose, and swan). Additionally, we examined the references in the source papers we collected to obtain additional studies containing relevant phylogenetic information.

The protocol for inclusion or rejection of source trees was guided by the issues of data quality (e.g. data independence and duplication, see Gatesy *et al* 2002) following the principles described in Bininda-Emonds *et al* (2004) and as implemented in Beck *et al* (2006). Generally, only trees that were based on an actual analysis of a novel, independent data set were collected

for our analysis. Reasons for the exclusion of potential source trees included the lack of any explicit underlying data set (e.g. as for taxonomies), the simple replication of the results of previous studies without any novel analysis, or an insufficient number of Galloanserae species for the tree to be phylogenetically informative in the context of this study. All nonindependent trees were retained at this stage, with corrections for any nonindependence being applied subsequently (see below). Nonindependence could arise both between studies (e.g. through use of the same data set on an overlapping species sample) and/or within the same study (e.g. multiple analyses of the same data set).

A total of 400 phylogenetic trees derived from molecular and/or non-molecular (e.g. morphological or behavioral) data, and obtained using distance (e.g. neighbor-joining) or character-based methods (e.g. parsimony, maximum likelihood, and Bayesian analysis) was included initially as source trees. A topology equivalent to the classification of Dickinson (2003) was also included as a "seed tree" to increase taxonomic overlap among source trees while providing only limited and usually uncontroversial phylogenetic information. The use of seed trees has been shown to improve the resolution of the supertree and to decrease computation time in simulation (Bininda-Emonds & Sanderson 2001) and when, suitably downweighted, does not distort the final topology compared to that dictated by the "real" source trees (see Beck *et al* 2006). All information in the source trees was coded and stored exactly as it appeared in the source publication (i.e. without any correction for apparent typos and/or synonyms in taxon names) into the tree window of MacClade (Maddison & Maddison 2000).

Standardization of Taxon Names

The set of 400 source trees, despite not including all extant species of Galloanserae, contained a total of 1368 taxon names because of the inclusion of numerous typos and synonyms (including the use of common names) for a given species (e.g. "Chicken" or "*Gallus gallus domesticus*" or "*Gallus gallus* 1" for *Gallus gallus*), of higher-level taxon names (e.g. *Gallus* or Galliformes), or of extinct species (e.g. the Turtle-jawed Moa-nalo, *Chelychelynechen quassus*) or of non-fowl species (e.g. the Rock Pigeon, *Columba livia*).

Therefore, where possible, the names of all terminal taxa were standardized to those in Dickinson (2003). Appropriate synonyms for unrecognized names were obtained primarily from the Integrated Taxonomic Information Service (ITIS: www.itis.gov) and secondarily from additional searches. All non-fowl species were synonymized to "outgroup" and higher-level terminal taxa were synonymized to the type species of the taxon (e.g. both *Gallus* and Galliformes were synonymized to *Gallus gallus*) following Bininda-Emonds *et al* (2004). Ambiguous names (e.g. "Basal Anseriformes and Galliformes", "Other Galliformes" or "Partridge") and extinct taxa were pruned from the source trees. Synonymization was achieved using the Perl script synonoTree v2.1 (Bininda-Emonds *et al* 2004). SynonoTree also accounts for cases where the process of synonymization yields non-monophyletic species by outputting all possible permutations of a given source tree where each such species is represented only once in each of its possible placements. Finally, all trees containing the taxon "outgroup" were rooted on this taxon, which was subsequently deleted. All other source trees were held to be unrooted. Trees that were synonymized so as to become phylogenetically uninformative (i.e. containing less than three or four species for rooted and unrooted trees, respectively) were deleted, as were any completely unresolved trees. Altogether the synonymization process reduced the number of

source trees to 385 (from 110 published studies; including the seed tree) and 43 trees that represented additional permutations of 31 source trees.

MRP Supertree Construction

Supertree construction used MRP, which represents by far the best investigated and most frequently used supertree method (Bininda-Emonds 2004). MRP operates by coding the topology of a tree as a series of binary pseudocharacters, each pseudocharacter representing one informative node in the tree. Taxa derived from the node are scored as 1, those that are not, but are still present on the tree are scored as 0, and taxa present only on other trees in the entire set are scored as ?. The matrix representations of each tree are then combined into a single matrix for parsimony analysis. Normally an all-zero outgroup is added to the matrix. However, we used semi-rooted MRP coding (Bininda-Emonds *et al* 2005) as implemented in the Perl script SuperMRP v1.2.1 in which the outgroup was scored with zeros only for rooted trees; for unrooted trees, it was scored as ?.

The final MRP matrix consisted of 4713 pseudocharacters that were differentially weighted across trees to account for source-tree nonindependence, whether at the level of the underlying data or because of permutations of a given tree arising from non-monophyletic taxa. The source trees were initially subdivided according to data type, with sets of nonindependent studies within each category being determined on a case-by-case basis: mixed-data analyses (five sets for seven trees), molecular data (83 sets for 236 trees), morphological data (one set for 59 trees), other data types (13 sets for 22 trees), and unspecified data (13 sets for 13 trees). Weighting was applied in a hierarchical fashion, first according to data-set nonindependence and then to permutation nonindependence. For example, pseudocharacters for each of the 59 trees in

the morphological data set received a weight of 0.017 (= 1/59). However, the pseudocharacters for the morphological study of Livezey (1991) were downweighted by an additional factor of two beyond this (to 0.008) to account for the two permutations of this tree generated by synonoTree. Finally, the seed tree of Dickinson (2003) was given a weight of 0.001 (= at least six times smaller than any other source tree) to minimize its impact on the supertree topology beyond helping to stabilize the analysis.

Parsimony analysis used PAUP* v4.0b10 (Swofford 2002) and employed a parsimony ratchet (Nixon 1999) consisting of 50 batches of 200 replicates initially, followed by a brute force search using all optimal trees found to that point as starting trees. During the reweighting steps, 25% of the MRP pseudocharacters were selected at random and given a weight of two before being returned to their initial differential weights. Starting trees for each batch were obtained using a single random-addition sequence. All searches used TBR branch-swapping. Ratchet searches allowed only a single tree to be retained at any given step, whereas the terminal brute-force search allowed multiple trees. All instructions for the ratchet were produced by the Perl script perlRat v1.0.9 and implemented in PAUP* as a paup block. The initial ratchet analysis saved a maximum of 10,050 equally most parsimonious trees. These trees then served as the starting trees for the extended brute-force search saving up to 100,000 trees. The strict consensus trees from the initial and ratchet and subsequent brute force searches were identical, hinting that the ratchet had reached a form of "convergence" in that the additional equally most parsimonious solutions showed conflict with existing areas of incongruence rather than generating new conflict (and thereby decreasing resolution). The final supertree was held to be the strict consensus of the set of 100,000 equally most parsimonious solutions (each of length

1418.607). Both it and a majority-rule consensus of the same set of trees have been deposited with TreeBASE (Sanderson *et al* 1994).

Differential support within the supertree was determined using the rQS index as implemented in QualiTree v1.2.1 (Bininda-Emonds 2003; Price *et al* 2005), which measures the amount of support and disagreement for a given node in the supertree among the set of source trees. As such, it avoids the inherent nonindependence between MRP pseudocharacters, which violates the assumptions underlying such conventional support measures as the bootstrap (Felsenstein 1985a) or Bremer support (Bremer 1988) and causing them to be invalid in this context. An rQS value varies between +1 and –1, indicating that all sources trees support or contradict the nodes in question, respectively. Empirically, rQS values usually tend to be slightly negative (e.g. Price *et al* 2005; Beck *et al* 2006), reflecting the fact that many phylogenies are uninformative for a given node (thereby scoring zero for it) and those that are informative tend to conflict with one another, even if slightly. Therefore, even slightly positive rQS values should be taken to indicate good support. All Perl scripts used in this study are available from http://www.uni-oldenburg.de/molekularesystematik/33997.html.

RESULTS AND DISCUSSION

Taxonomic Coverage and Resolution

Our fowl supertree includes 376 species, comprising over 83% of all 452 fowl species recognized by Dickinson (2003) (Table 1). All 162 Anseriformes species and 74% of all 290 Galliformes species are present in the supertree. The distribution of the 110 studies yielding source trees shows that the number of phylogenetic studies for fowls has increased rapidly since the late 1980s, with a sharp increase in particular for studies using molecular data, either alone or

in combination with morphological or other data sources (Figure 2.1). Overall, Galloanserae are relatively well characterized phylogenetically. The number of source trees per fowl species present in the tree (1.0) was more than that in supertrees of well-studied mammalian groups of comparable size [e.g. 0.6 in primates or bats (Purvis 1995; Jones *et al* 2002), and 0.7 in carnivores (Bininda-Emonds *et al* 1999)], despite our more conservative source tree inclusion protocol. The value continues to exceed those of the mammalian supertrees even when we calculate it for all extant species, including those not present on the tree (0.83) to make it comparable to the mammal values.

The supertree highlights that phylogenetically poorly-characterized species often tend to be clumped: the majority of species missing in the supertree are assigned to Odontophoridae (59% missing), Cracidae (32% missing), and Phasianidae (20% missing). The uneven distribution of missing species often appears associated with issues of geography and/or accessibility of the species. For example, species of the genus *Odontophorus*, which represents almost half of all species in Odontophoridae (15 of 32), are found in Neotropical forests, but the genus is represented by only a single species (*Odontophorus gujanensis*) in the supertree. Similarly, only a single species out of the 20 in *Arborophila* (*Arborophila torqueola*), which generally inhabit Southeast Asian tropical forests or high alpine meadows in the Himalayas and often in widely scattered populations, was present in the supertree. Obviously, deriving a complete phylogenetic estimate of Galloanserae will require an increase in future research effort towards these and other missing species.

Although the limit of 100,000 equally most parsimonious solutions was reached, the strict consensus of them was well resolved, containing 304 of a maximum possible 375 nodes (= 81.1%; Table 1). This degree of resolution was higher than that for many other supertrees of

comparable scale, including those for primates (79%; Purvis 1995), carnivores (78%; Bininda-Emonds *et al* 1999), marsupials (74%;Cardillo *et al* 2004), bats (46%; Jones *et al* 2002), whale and even-toed hoofed mammals (60%; Price *et al* 2005), shorebirds (50%; Thomas *et al* 2004), and seabirds (63%; Kennedy & Page 2002). Again, the degree of resolution varied across the tree and among the families in particular, ranging from 61% for Cracidae to 100% for Anhimidae and Numididae. Cases of decreased resolution among and within families appear to derive more from a lack of agreement among the source trees than from a lack of available information. Even less resolved families contained a large amount of available data. For example, there were 417 pseudocharacters per species for the 61%-resolved Cracidae, compared to 373 for the 94%-resolved Megapodiidae. Additionally, the majority-rule consensus fowl supertree indicates increased resolution of 88% for Cracidae, 98% for Anatidae, and 96% for all fowl species examined. The occurrence of the poorly resolved groups in the supertree also highlights areas in need of more rigorous systematic analyses in the future.

To date, the most comprehensive phylogenies for Anseriformes and Galliformes (Livezey 1997 and Crowe *et al* 2006, respectively) have been at the genus- and not species levels. These trees necessarily assume the monophyly of each genus, often forcing the wide range of ecological and evolutionary hypotheses that have been examined using these trees to be based on the average of the respective biological characters of the constituent species (e.g. Keane *et al* 2005; Kolm *et al* 2007). Crucially, however, our species-level supertree showed that more than 30% of the polytypic genera included were not monophyletic (8 of 18 anseriform and 9 of 35 galliform genera). This suggests that the results from the genus-level comparative studies using the average of the species' traits should be interpreted with caution until analogous species-level comparative studies are available.

Anseriformes-Galliformes Relationships

The supertree supported the monophyly of each of the orders Anseriformes and Galliformes (Figures 2.2-2.3), reflecting historical agreement on this point (but see Prager & Wilson 1976). In addition, both clades enjoyed high support as measured by the rQS index (0.390 for Anseriformes and 0.655 for Galliformes; see Appendix I), meaning that monophyly was directly specified by the majority of relevant source trees in each case.

Anseriformes

The supertree supported the partitioning of Anseriformes into the three traditional families (Figure 2.2) Anhimidae (screamers), the monotypic Anseranatidae (Magpie Goose), and Anatidae (ducks, geese, and swans). Anatidae was the sister group to the two other families, which was consistent with DNA-DNA hybridization (Sibley & Ahlquist 1990), and nuclear and mitochondrial DNA studies (e.g. Sorenson *et al* 2003). This resolution, however, conflicted with some morphology-based topologies (e.g. Livezey 1997) and nuclear DNA studies (e.g. RAG-2 exon; see Cracraft *et al* 2004), where Anhimidae formed the sister group. This uncertainty was also reflected in the slightly negative rQS value (-0.044) for the clade containing both Anhimidae and Anseranatidae.

Based on behavioral patterns, Delacour and Mayr (1945) split Anatidae into the two subfamilies Anserinae and Anatinae, a pattern followed by del Hoyo *et al* (1992). This classification was amended recently by Livezey (1997) and Dickinson (2003), who each recognized five subfamilies, splitting Dendrocygninae and the monotypic Stictonettinae (Freckled Duck) from a redefined Anserinae, and Tadorninae from Anatinae. However, the

supertree did not provide strong support for either scheme, with only Anserinae *sensu* Livezey (1997) and Dickinson (2003) being found to be monophyletic within a paraphyletic Anatinae.

The supertree revealed a paraphyletic Dendrocygninae with respect to the remaining Anatidae, placing it as the first group to evolve in Anatidae. This basal position of the subfamily reflected the majority of the source topologies (e.g. Sibley & Ahlquist 1990; Livezey 1997). However, the internal relationships of Dendrocygninae in the supertree contradicted most traditional taxonomic groupings, including the monophyly of *Dendrocygna* (whistling ducks) and its sister group relationship with *Thalassornis*.

The relative position of Stictonettinae also differed among the source references. Various authors have linked it with any of Dendrocygninae (Woolfenden 1961), Anserinae (Johnsgard 1965), or Tadorninae/Anatinae (Livezey 1997) based on morphological or behavioral characters. Our study also reflected this uncertainty, placing it in a polytomy with all other subfamilies.

Anserinae monophyly has been supported by both morphological (e.g. Livezey 1997) and molecular studies (e.g. Donne-Gousse *et al* 2002), a fact reflected in our supertree (rQS = 0.052), with 26 source trees supporting its monophyly and only six contradicting it. Resolution within Anserinae was complete and each of the three polytypic genera recognized by Dickinson (2003) (*Anser*, *Branta*, and *Cygnus*) were recovered as monophyletic. *Anser* and *Branta* formed a clade (rQS = 0.044; 21 source trees in agreement and only four in conflict), consistent with the majority of studies recognizing them as the tribe Anserini (true geese, e.g. Livezey 1997). However, disagreement among the source trees about the interrelationships of *Cygnus*, *Coscoroba* and *Cereopsis* lead the relative position of these genera being somewhat equivocal in the supertree (rQS = -0.008 for the clade as a whole and rQS = 0.000 for the grouping of

Coscoroba and *Cereopsis*). For example, a morphological study (Livezey 1997) recognized the clade of *Cygnus* + *Coscoroba* as the tribe Cygnini (swans), and *Cereopsis* as the independent tribe Cereopsini, which was regarded as a distant relative to *Cygnus* + *Anser* + *Branta*. However, a recent molecular study placed *Cereopsis* and *Coscoroba* as sister genera, with *Cygnus* as sister to this clade (Donne-Gousse *et al* 2002), as was found in this study. This latter branching pattern is also congruent with the disjunctive geographical origins of the genera, with *Cygnus* originating in the Northern Hemisphere and the other two genera coming from the Southern Hemisphere (Donne-Gousse *et al* 2002).

Strong disagreement exists with respect to the compositions of and interrelationships between Tadorninae and Anatinae, which is reflected in the supertree by neither subfamily being recovered as monophyletic. Nor do the two subfamilies form a clade (although the majority of their members do cluster together), with Anserinae embedded within them. For instance, whereas Dickinson (2003) did not delineate any tribes for the subfamilies in his classification, del Hoyo *et al* (1992) divided Tadorninae + Anatinae into eight tribes. Independently of this, Livezey (1997) also divided Tadorninae into three tribes and Anatinae into five tribes. However, despite the similar numbers of tribes erected by these two authors, few are identical in terms of their composition (e.g. Tadornini, comprising *Tadorna*, *Chloephaga*, *Neochen*, *Alopochen*, and *Cyanochen*). Instead, different compositions are the rule. For example, whereas Livezey (1997) included *Hymenolaimus* in Merganettini (Tadorninae), del Hoyo *et al* (1992) considered it to be part of Anatini (Anatinae).

This supertree reflected these disagreements, with only the tribe Malacorhynchini (comprising *Malacorhynchus* and *Salvadorina*) being recovered unequivocally as monophyletic (Tadornini was monophyletic in the majority-rule supertree), and then strongly so, with ten

source trees supporting the clade and none opposing it (rQS = 0.026). Moreover, whereas Malacorhynchini formed a clade with Oxyurini (*Heteronetta*, *Biziura*, *Nomonyx*, and *Oxyura*, but also unconventionally including *Nettapus*), this clade was positioned as part of a polytomy with Anserinae (or basal to it in the majority-rule supertree), hinting at the possible non-monophyly of Tadornine + Anatinae. Again, however, this uncertainty simply reflects historical disagreement. For example, the DNA-DNA hybridization study of Sibley and Ahlquist (1990) placed the *Oxyura* as sister to the remaining Anatidae, which is broadly consistent with our results, but Malacorhynchini in Anatinae, and therefore not directly related to *Oxyura*. By contrast, morphological evidence (e.g. Livezey 1997) tends to place Malacorhynchini at the base of the whole Anatinae. Thus, the relative positions of Malacorhynchini and Oxyurini appear to differ between molecular and morphological data. This conflict was also reflected in the rQS value of 0.000 for the relationship between Malacorhynchini and its sister clade, with 17 source trees in agreement and another 17 source trees in disagreement with this arrangement.

Resolution within the remaining members of Tadorninae and Anatinae (which formed a clade) was generally poor, with the clade displaying a large basal polytomy and the poor resolution also extending from the tribal-level down through the genus- and species-levels. Only 46% (6 of 13) of the polytypic genera within Tadorninae + Anatinae were monophyletic in the supertree, and the entire clade was less than 70% resolved. The majority-rule supertree reveals better overall resolution for this clade (97%), and at the species- and the genus-levels in these subfamilies in particular. Resolution, however, remained poor at the higher taxonomic levels.

Galliformes

Traditionally, the relative positions between Megapodiidae (megapodes) and Cracidae (chachalacas, curassows, and guans), and among Numididae (guineafowls), Odontophoridae (New World quails), and Phasianidae (partridges, turkeys, grouse, and pheasants) have been contentious. Some authors suggested a sister-group relationship between Megapodiidae and Cracidae, designating them as the superfamily Cracoidea (Wetmore 1960), the suborder Craci (del Hoyo *et al* 1994), or even as the independent order Craciformes (Sibley & Ahlquist 1990). However, more recent phylogenies based on morphology (e.g. Dyke *et al* 2003), molecular data (e.g. Dimcheff *et al* 2002) or their combination (e.g. Crowe *et al* 2006) all tend to support Megapodiidae as being sister to the remaining Galliformes (including Cracidae), with Cracidae then being sister to the remaining forms. Our majority-rule supertree broadly reflected this latter pattern, supporting the sequential sister-group relationships of Megapodiidae and Cracidae (with the exception of *Ortalis vetula*, thereby making Cracidae non-monophyletic), and the remaining Galliformes; these groups formed part of a large polytomy in the strict-consensus supertree (Figures 2.2-2.3).

Our supertree supported Numididae as being sister to the remaining families Odontophoridae and Phasianidae, with the clade comprising all three families having a high rQS value of 0.566. This arrangement agrees with those derived from nuclear (e.g. Armstrong *et al* 2001), mitochondrial (e.g. Dimcheff *et al* 2002), and combined morphological and molecular data (e.g. Crowe *et al* 2006). That being said, the position of Odontophoridae remains largely unresolved. For example, recent phylogenetic trees derived from DNA-DNA hybridization (e.g. Sibley & Ahlquist 1990), morphological (e.g. Dyke *et al* 2003), and combined morphological and molecular data (e.g. Crowe *et al* 2006) place the family in a variety of positions within

Phasianidae. Our supertree follows suit and recovers Odontophoridae as a relatively basal group within Phasianidae. However, it is noteworthy that most phylogenetic studies have included only a few species of Odontophoridae, such that we lack robust phylogenetic information for more than half of all species of this family. Thus, the relative position of Odontophoridae indicated here should likewise be regarded as tentative and should be revisited in the future with increased taxon sampling.

The monophyly of Megapodiidae was supported in the supertree (rQS = 0.081) and relationships within the family were largely congruent with several traditional species-level phylogenies (e.g. Jones *et al* 1995; Birks & Edwards 2002; Crowe *et al* 2006). Support for the monophyly of the genus *Megapodius* in particular was strong, with 10 source trees supporting it and none directly opposing it (rQS = 0.026). *Macrocephalon* was recovered as the sister to the clade of *Eulipoa* + *Megapodius*, albeit with equivocal support (rQS = 0.000). Monophyly of *Aepypodius* was not supported.

The source trees did not support Cracidae monophyly absolutely, although the family is monophyletic in the majority-rule supertree (and found in 94% of all 100,000 equally most parsimonious solutions). Much of the conflict can be traced to the historical uncertainty regarding the two genera *Oreophasis* and *Ortalis*, which have been placed within either Cracinae (e.g. Crowe *et al* 2006) or Penelopinae (e.g. del Hoyo *et al* 1994; Dickinson 2003). The strict-consensus supertree makes no definitive statement to resolve this conflict; however, the majority-rule supertree suggests that the affinities of the two genera lie with Cracinae. However, *Ortalis* was not recovered as monophyletic in either supertree. Beyond this, the subfamilies Cracinae (curassows) and Penelopinae (chachalacas and guans) were found to be monophyletic, although the degree of resolution within each varied considerably. Support for Cracinae was

strong, with 28 source trees directly supporting and none directly contradicting it (rQS = 0.073). By contrast, relationships within Penelopinae were unclear, largely because of the non-monophyly of *Penelope*.

There was strong support for the monophyly of Numididae, which was directly supported by 20 source trees and contradicted by only two (rQS = 0.047). The species-level relationships in the family were completely resolved and each of the two polytypic genera (*Agelastes* and *Guttera*) was monophyletic. The branching pattern within the family disagreed with that presented by Crowe (1978), but was identical to that based later on combined morphological and molecular data (Crowe *et al* 2006).

Similarly, monophyly of Odontophoridae was also well supported, being present in 52 source trees and contradicted by only a single tree (rQS = 0.132). Relationships within the family were largely consistent with those based on a wide range of data types, including osteological (e.g. Holman 1961), ecological (e.g. Johnsgard 1983), allozyme (e.g. Gutierrez *et al* 1983), and combined morphological and molecular data (e.g. Crowe *et al* 2006). *Philortyx fasciatus* has been grouped traditionally with some genera adapted to the forest edge, such as *Colinus*, *Callipepla*, and *Oreortyx* (e.g. Holman 1961; Johnsgard 1983), but our supertree placed it as sister to the remaining Odontophoridae. Again, however, this relationship, and all other relationships within the family, should be interpreted with some degree of caution given the poor phylogenetic sampling effort in the family to date.

Within a polyphyletic Phasianidae, sequential sister-group relationships of the four subfamilies Perdicinae (partridges), Meleagridinae (turkeys), Tetraoninae (grouses), and Phasianinae (pheasants) were broadly recovered in the supertree, albeit with some exceptions. The supertree revealed seven subdivisions of Perdicinae, six of which were monophyletic. The

first was a paraphyletic assemblage of *Rhizothera* and the monotypic genera *Galloperdix*, *Ptilopachus*, *Haematortyx*, and *Melanoperdix* situated basal to Odontophoridae and the remaining Phasianidae. Among these genera, a sister-group relationship between *Galloperdix* and *Ptilopachus* was recovered, concurring with the results of Crowe *et al* (2006). The second group (rQS = 0.016) included *Xenoperdix*, *Rollulus*, *Arborophila*, and *Caloperdix*. The species composition and branching pattern within the group was in agreement with Crowe *et al* (2006), who designated this group as Arborophilinae. Similarly, the third group (rQS = 0.078) corresponds to Coturnicinae of Crowe *et al* (2006) and comprises Old World quails, the partridges *Coturnix* and *Alectoris*, and some *Francolinus* species. Relationships within *Coturnix* were unresolved, however, and its monophyly could also not be assured. The fourth group (rQS = -0.018) consisted of *Francolinus gularis, Francolinus pictus, Francolinus pintadeanus,* and *Francolinus francolinus*. In the fifth group, the monotypic *Bambusicola* formed a clade with the four species of *Gallus*. Although *Gallus* is typically allocated to Phasianinae, the grouping found in our supertree does find support in Crowe *et al* (2006), who named it Gallininae. In addition, the sister-group relationship between *Bambusicola* and *Gallus* was highly supported with an rQS value of 0.125. The sixth group (rQS = -0.026) consisted of the remaining *Francolinus* species, meaning that the supertree did not support the monophyly of the 41 species of *Francolinus*. Some authors, however, have suggested on the basis of morphological and molecular data that this genus be subdivided into at least five different genera (*Pternistis*, *Francolinus*, *Dendroperdix*, *Peliperdix*, and *Scleroptila*) (e.g. Crowe *et al* 1992; Crowe *et al* 2006). Although our results did not reflect these generic designations exactly, branching patterns within *Francolinus* and its relationships with other genera were largely congruent with those in Crowe *et al* (1992). The final group, the genus *Perdix* (rQS = 0.031), was placed as the sister taxon to

the clade of Meleagridinae + Tetraoninae, albeit with some uncertainty (rQS = -0.013), with 38 source trees contradicting this placement and 33 supporting it.

The sister-group relationship of Meleagridinae (two species in the genus *Meleagris*) and Tetraoninae was also not strongly supported (rQS = -0.021), although the monophyly of each showed better support (rQS = 0.016 and 0.119, respectively). Relationships within Tetraoninae were congruent with molecular (e.g. Gutierrez *et al* 2000; Dimcheff *et al* 2002; Drovetski 2002) and combined morphological and molecular data (e.g. Crowe *et al* 2006). The only exception was the position of *Lagopus*, with the low rQS value of the clade containing *Lagopus* and its sister group (-0.086) suggesting disagreement among the source trees.

The remaining Phasianinae (with the exception of *Gallus*) was split into the peafowl (e.g. *Pavo* and *Polyplectron*; rQS = -0.008) and pheasant groups (e.g. *Lophura* and *Tragopan*; rQS = 0.034) separated by the clade comprising *Perdix*, Meleagridinae, and Tetraoninae. Apart from this, the species composition and branching pattern within each group was highly congruent with phylogenetic trees based on molecular and morphological data (e.g. Crowe *et al* 2006).

CONCLUSION

Our supertree represents a first attempt to derive a comprehensive species-level phylogeny of Galloanserae, again highlighting the power of a traditional supertree approach (*sensu* Bininda-Emonds 2004) in this regard. Those areas where the supertree was either poorly resolved or incomplete tend to reflect gaps in the existing phylogenetic database (either ongoing disagreement and/or a lack of sufficient, robust phylogenetic information), and highlight areas in need of more study. Some of this missing information could perhaps be gleaned from taxonomies and other studies that are not based on the direct analysis of primary character data.

However, given that strong disagreement often exists within the studies we have included here, we felt it prudent not to include these additional sources. Like any phylogenetic hypothesis, our supertree is naturally open to further revision and resolution. In the meantime, however, it will provide a valuable foundation to understand the diverse biology of Galloanserae in a robust phylogenetic framework.

ACKNOWLEDGMENTS

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Table 2.1: Information for major clades of Gallanserae, including number of taxa recognized and covered in this study and summary statistics for the supertrees.

	No. of species recognized[a]	No. of species covered in this study	% coverage	% resolution		
				strict consensus	majority rule	rQS
Overall	452	376	83.2	81.1	96.3	0.037
Anseriformes	162	162	100	73.9	97.5	0.390
Anhimidae	3	3	100	100	100	0.091
Anseranatidae	1	1	100	.	.	.
Anatidae	158	158	100	72.6	97.5	0.366
Galliformes	290	214	73.8	86.9	95.8	0.655
Megapodiidae	22	17	77.3	93.8	93.8	0.081
Cracidae[b]	50	34	68	n/a	n/a	n/a
Numididae	6	6	100	100	100	0.047
Odontophoridae	32	13	40.6	91.7	100	0.132
Phasianidae[b]	180	144	80	n/a	n/a	n/a

[a] according to Dickinson (2003).

[b] Cracidae and Phasianidae were not monophyletic in the supertrees.

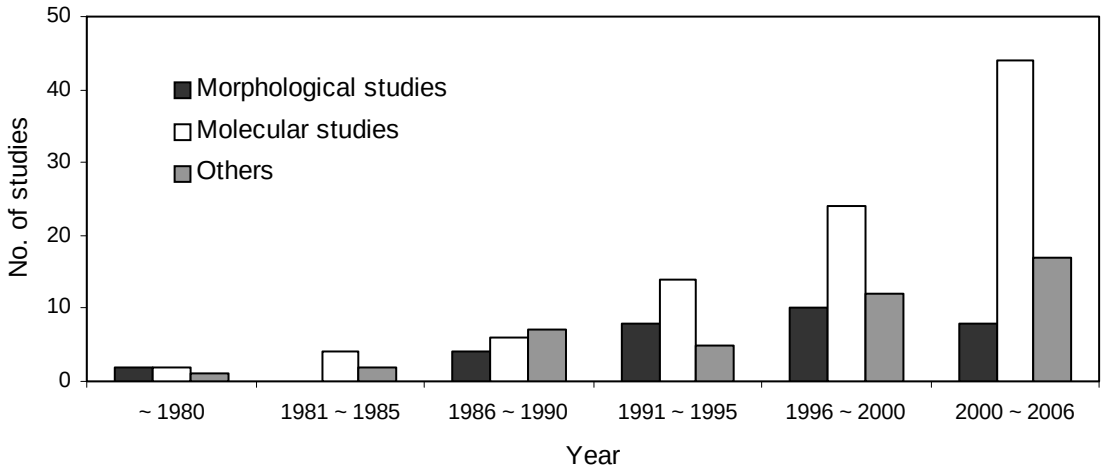

Figure 2.1: Temporal distribution of source trees included in the Galloanserae supertree.

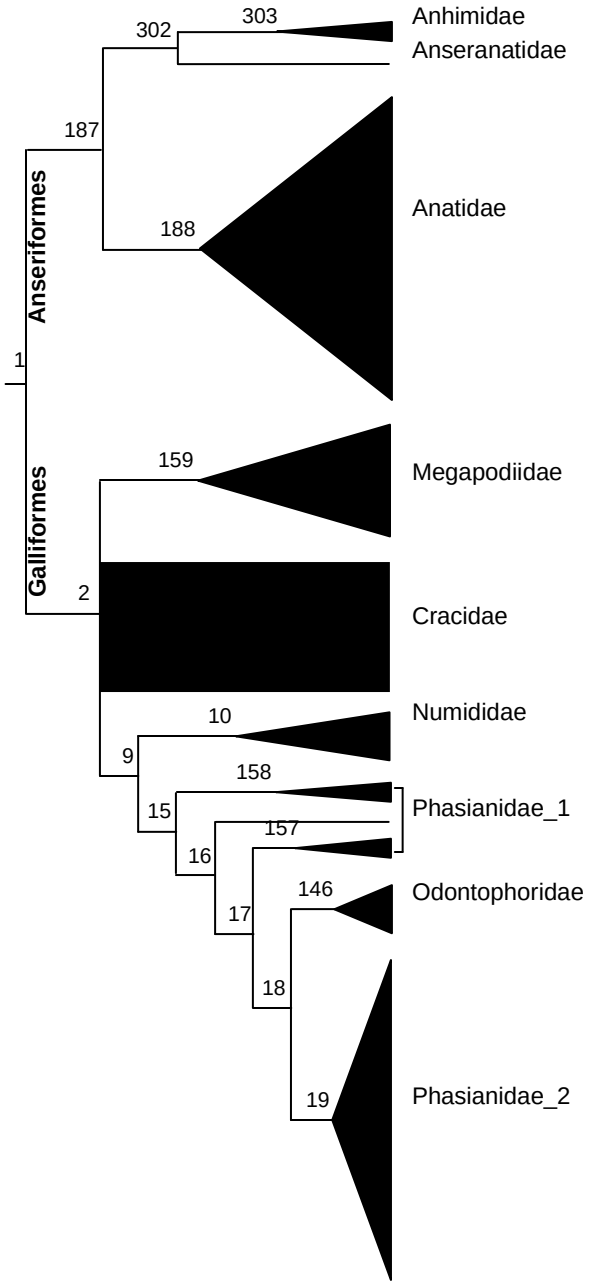

Figure 2.2: Partial representation of the Galloanserae supertree, showing interrelationships of and relative species richness of the major higher-level groups. Numbers on nodes represent node IDs (see Appendix A).

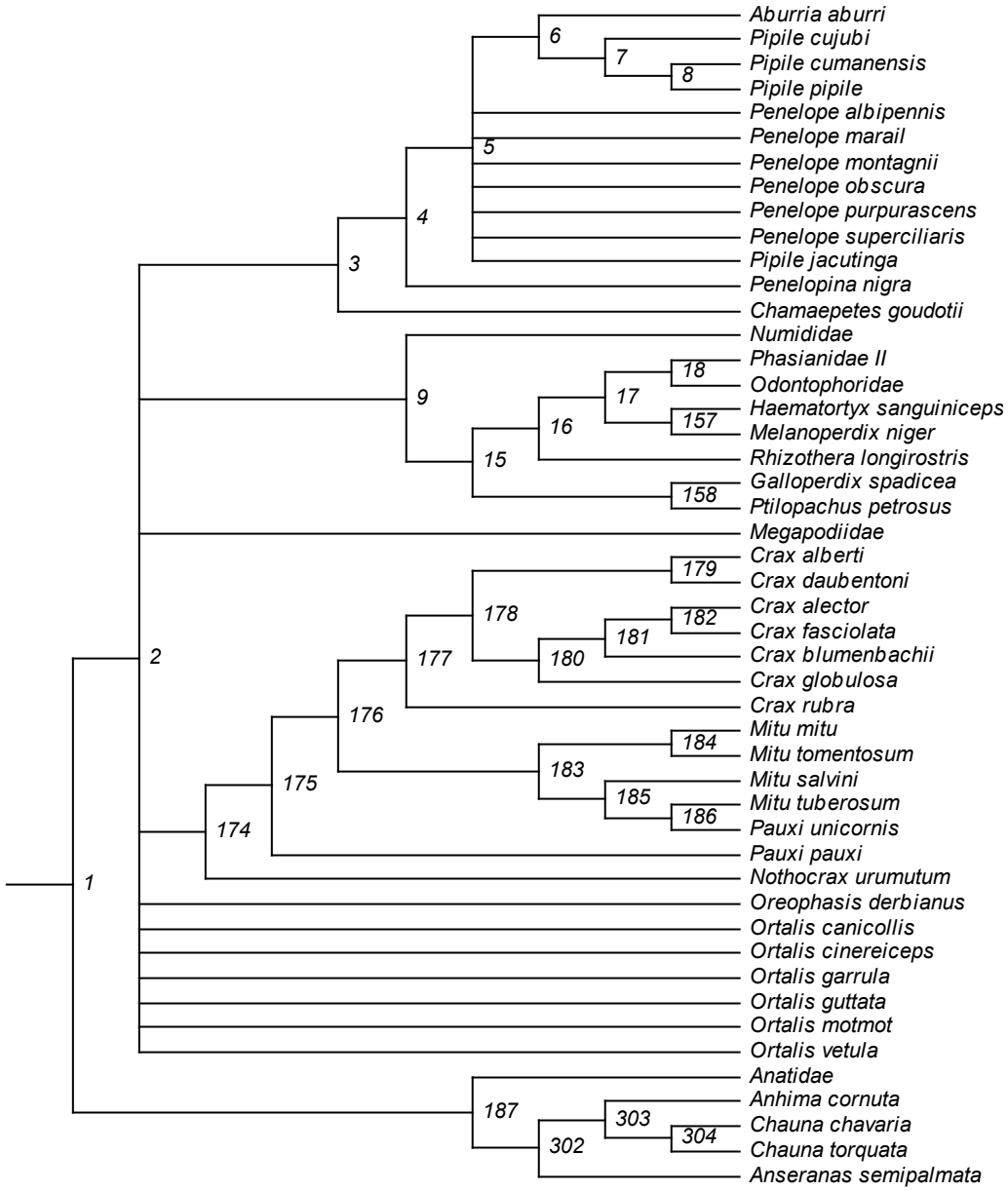

Figure 2.3: Component supertrees of the fowl supertree showing species-level relationships: A) Galloanserae.

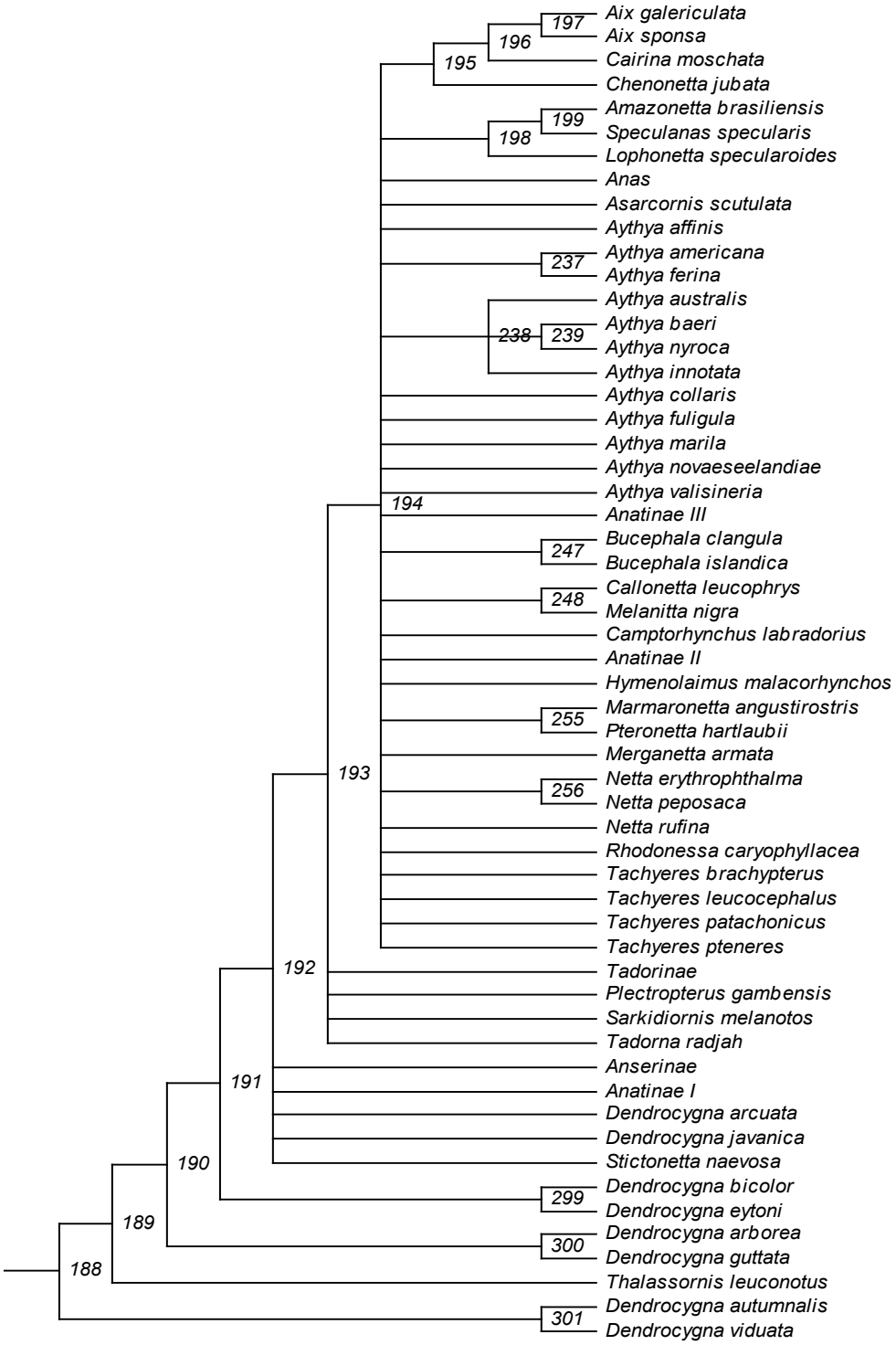

Figure 2.3: continued: B) Anatidae.

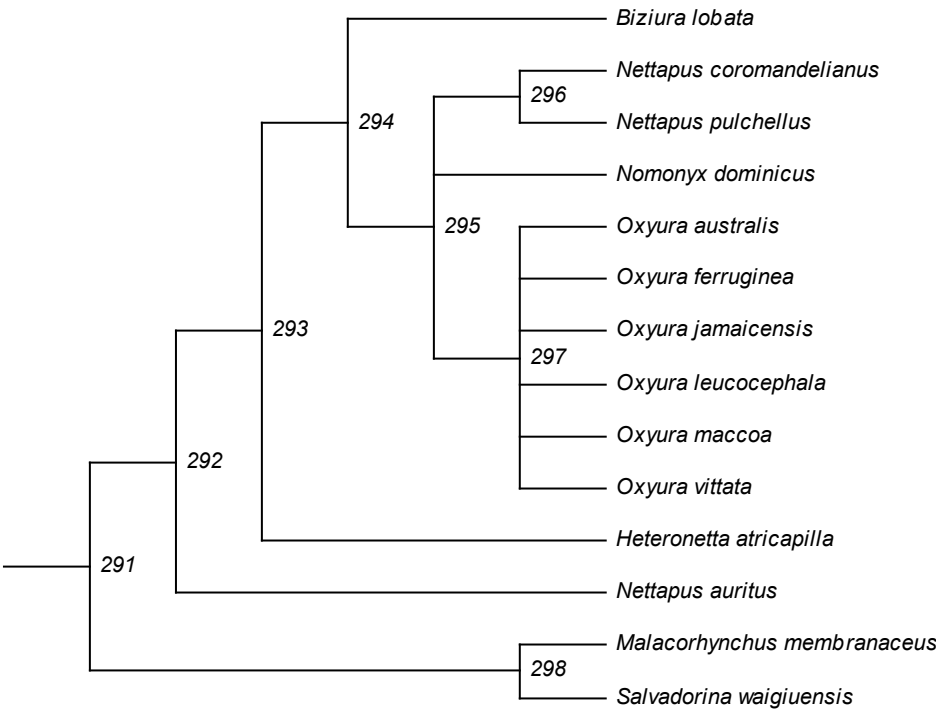

Figure 2.3: continued: C) Anatinae I.

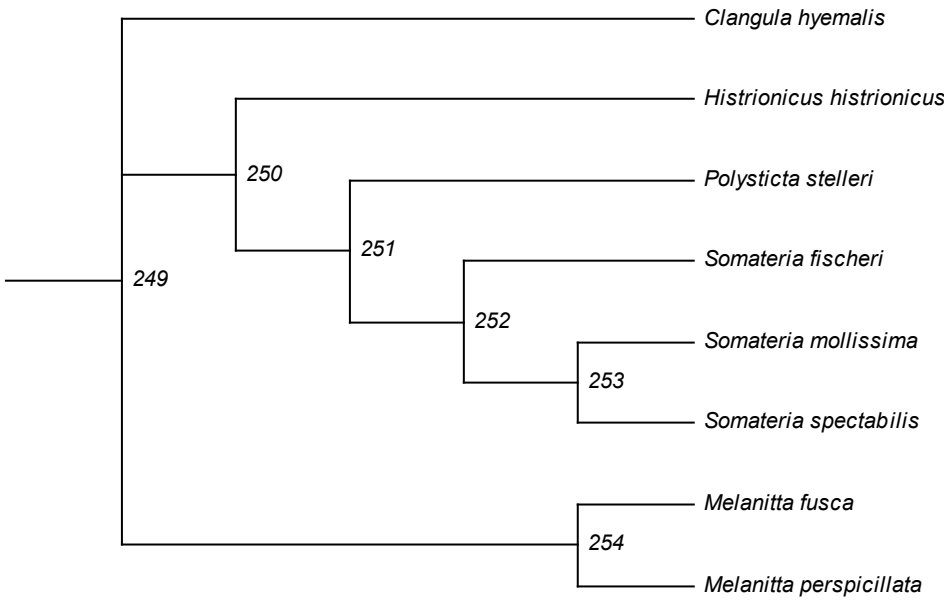

Figure 2.3: continued: D) Anatinae II.

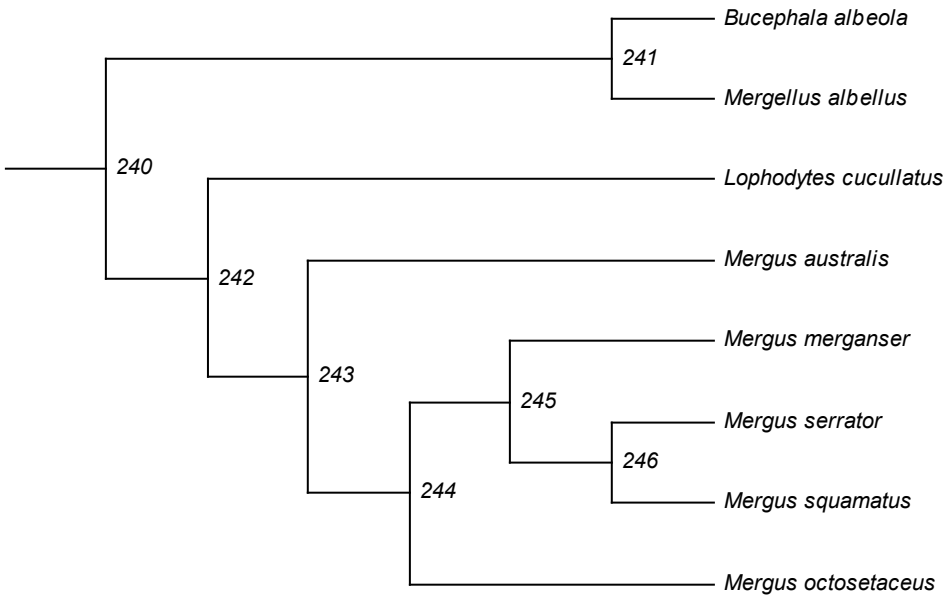

Figure 2.3: continued: E) Anatinae III.

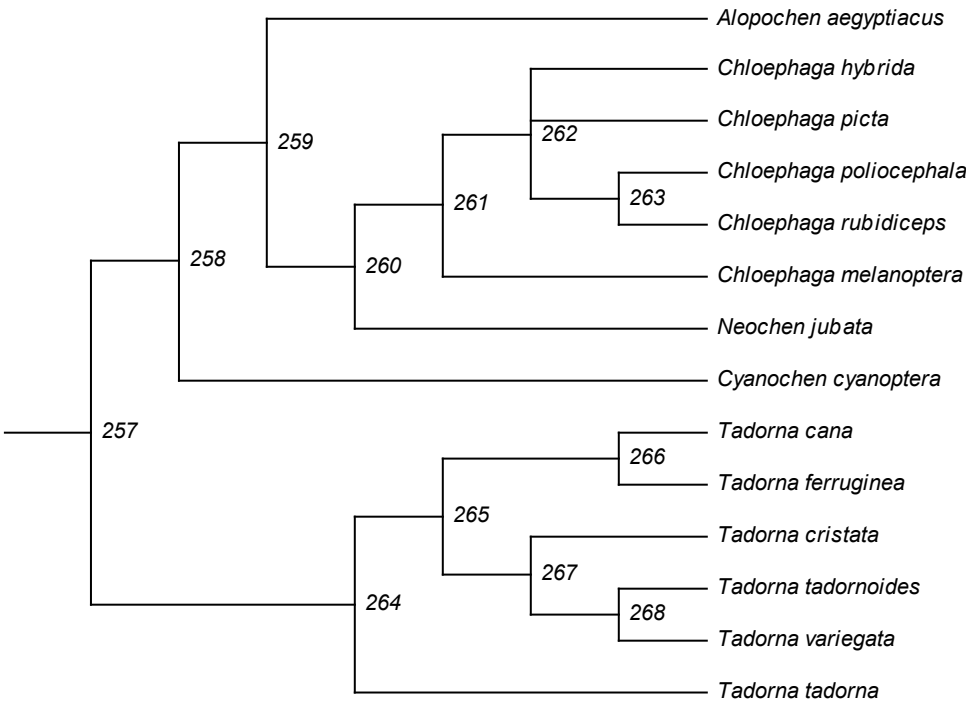

Figure 2.3: continued: F), Tadorinae.

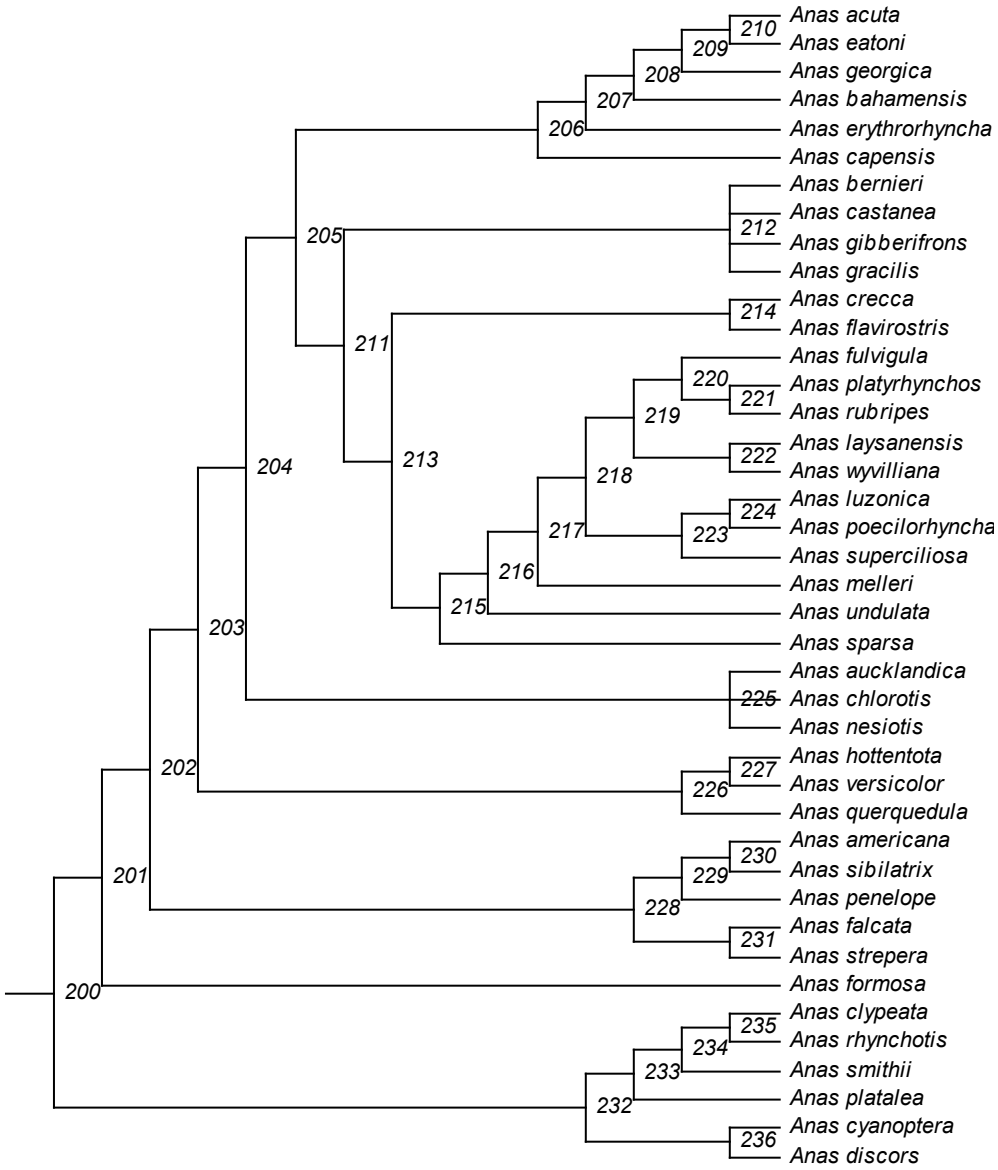

Figure 2.3: continued: G) *Anas*.

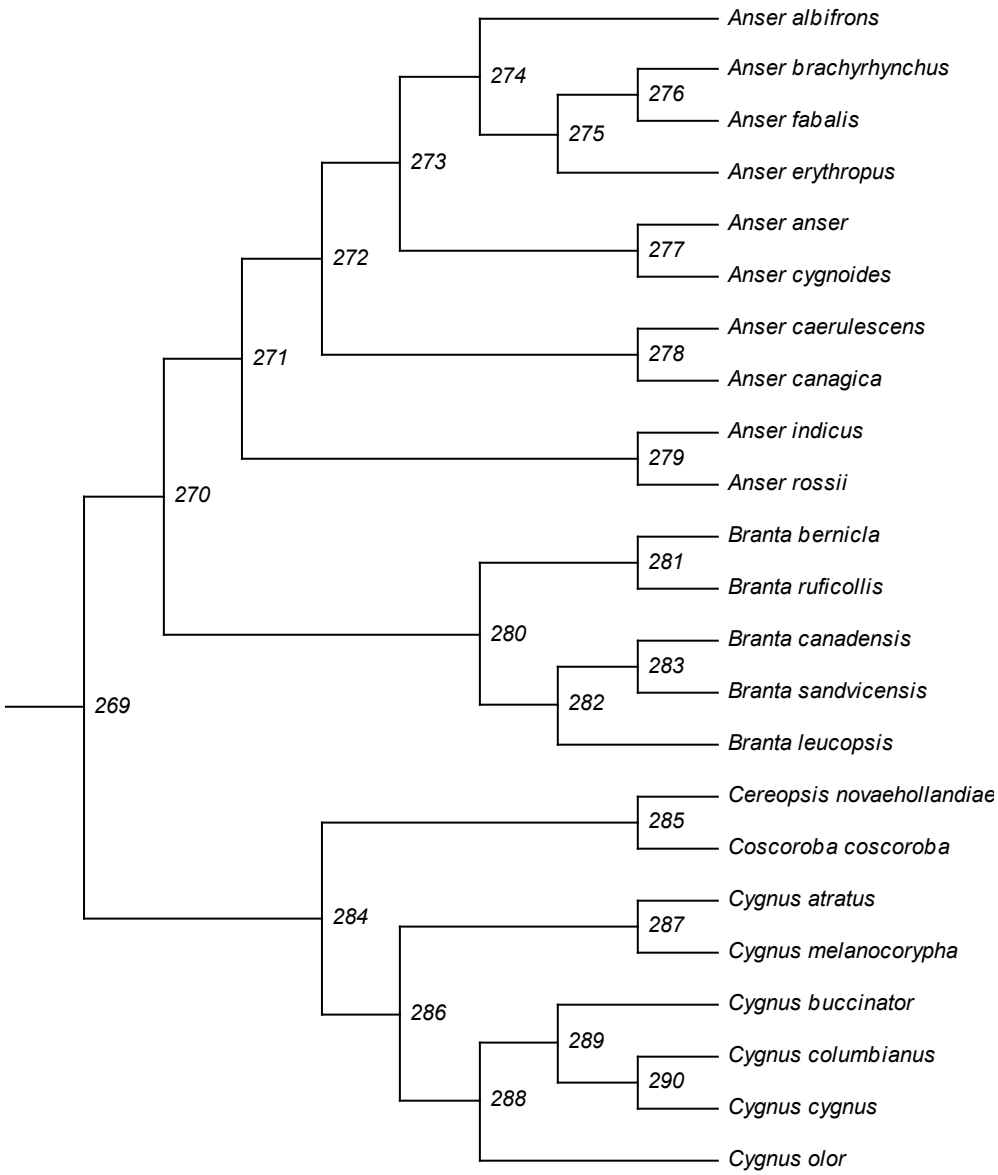

Figure 2.3: continued: H) Anserinae.

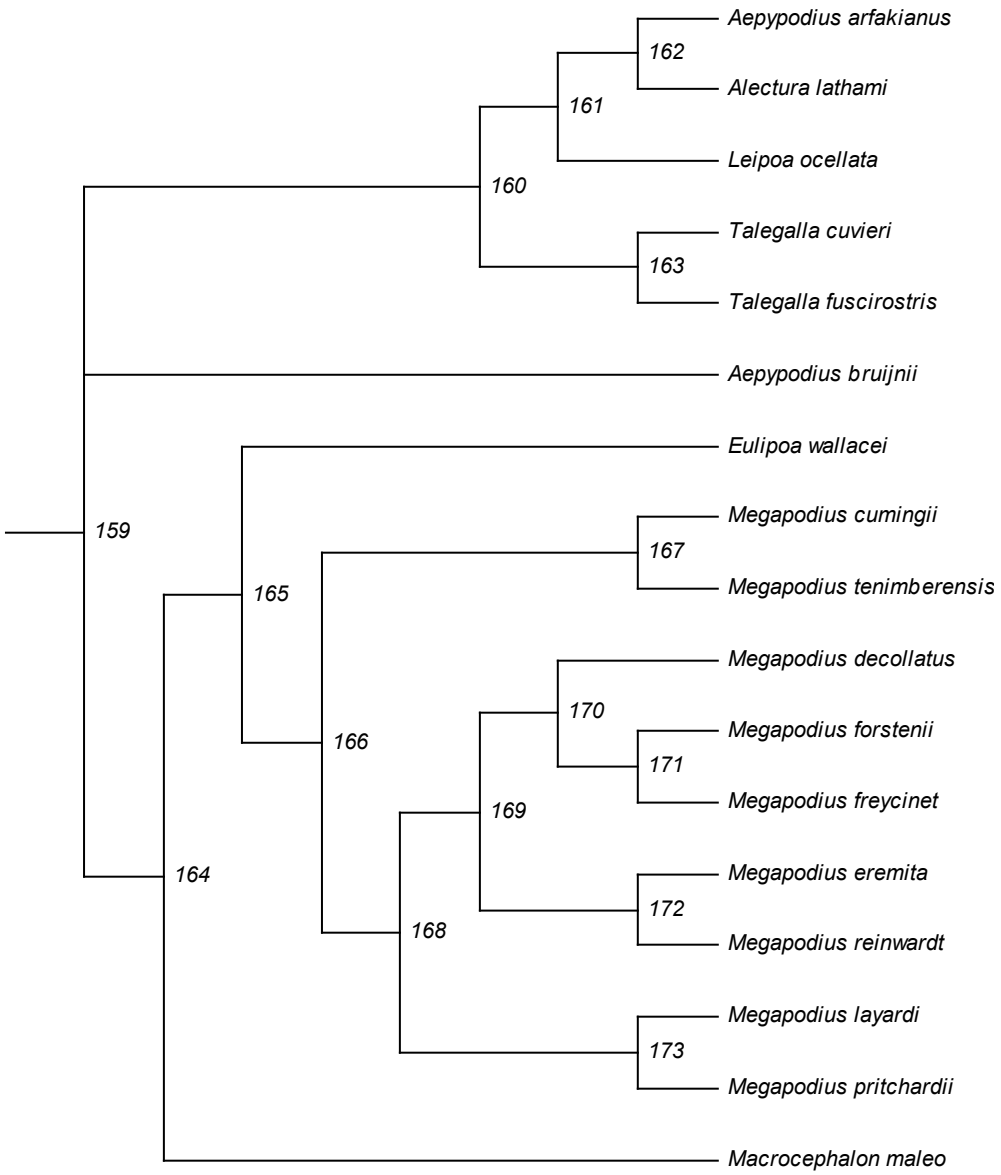

Figure 2.3: continued: I) Megapodiidae.

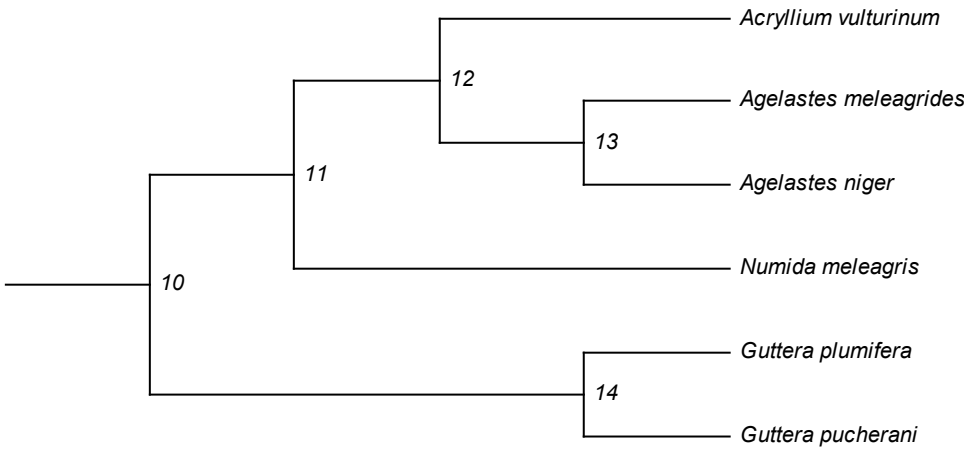

Figure 2.3: continued: J) Numididae.

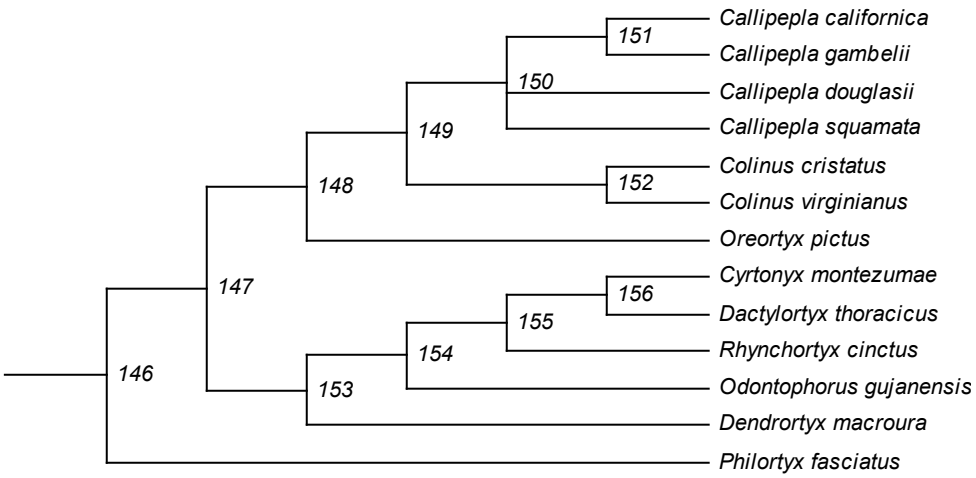

Figure 2.3: continued: K) Odontophoridae.

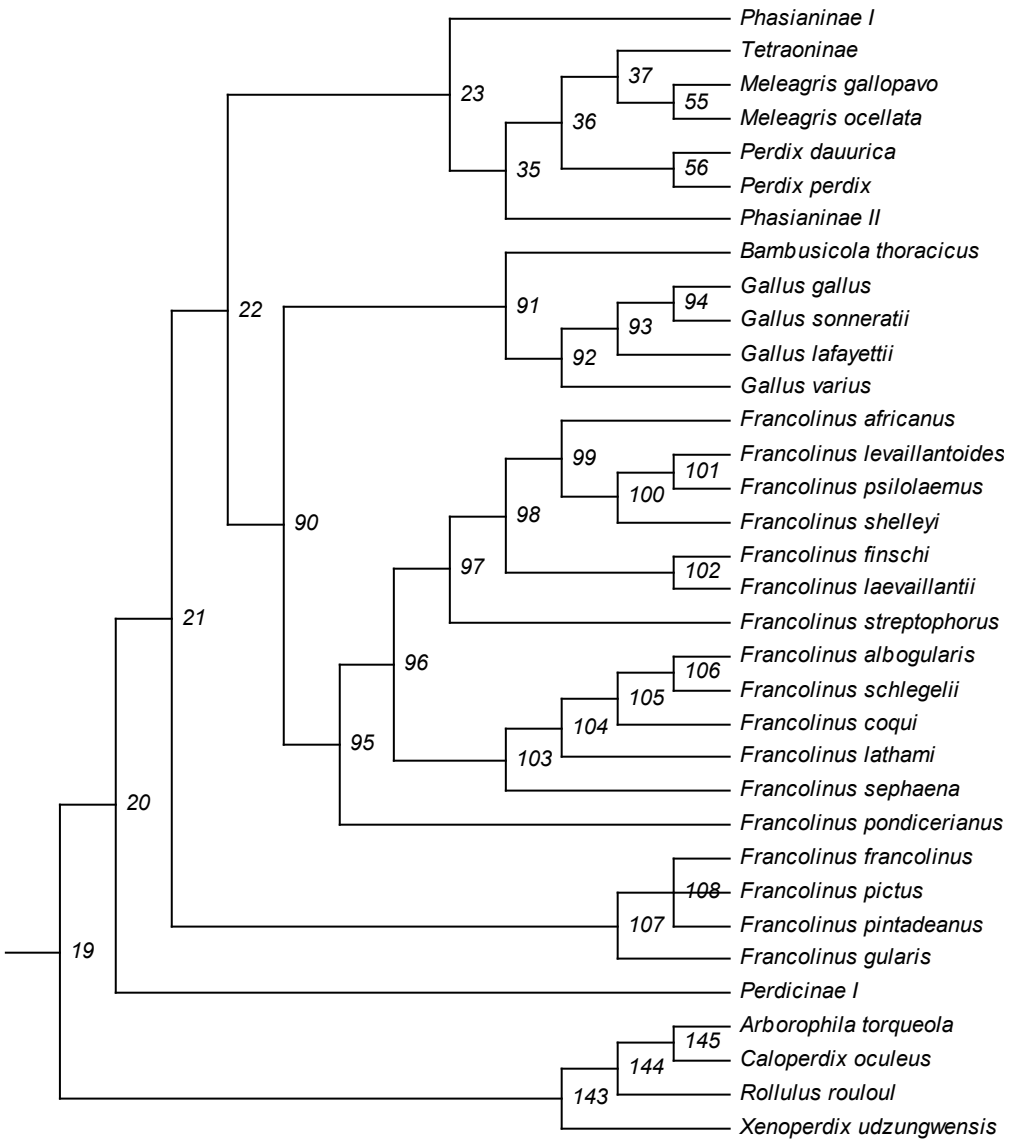

Figure 2.3: continued: L) Phasianidae II.

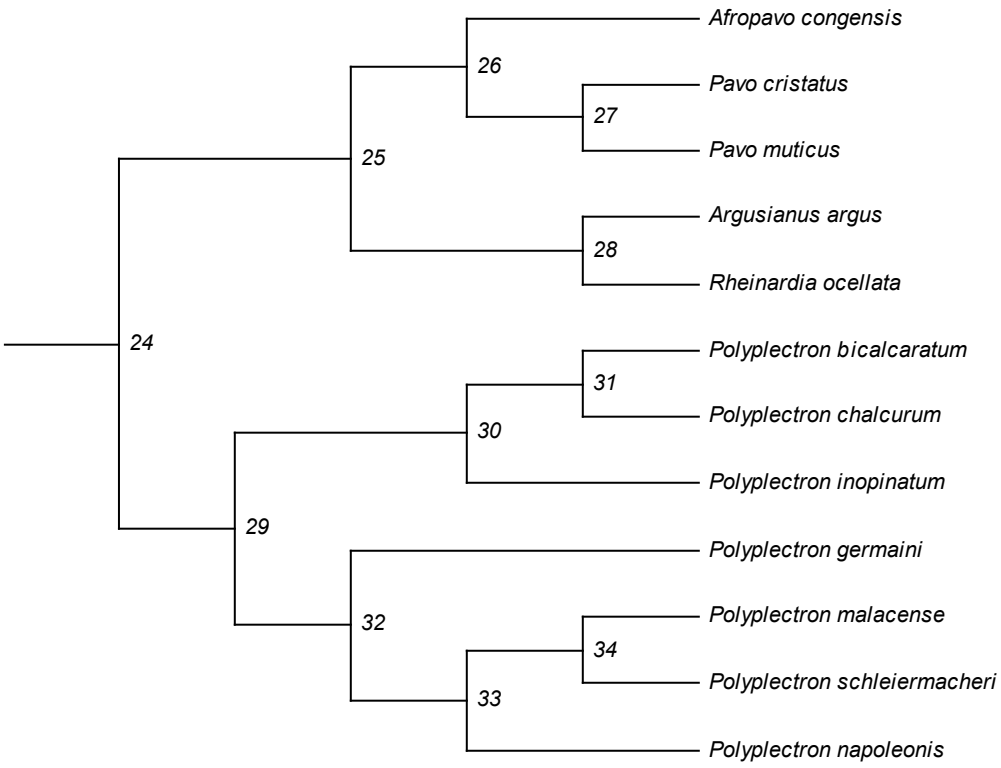

Figure 2.3: continued: M) Phasianinae I.

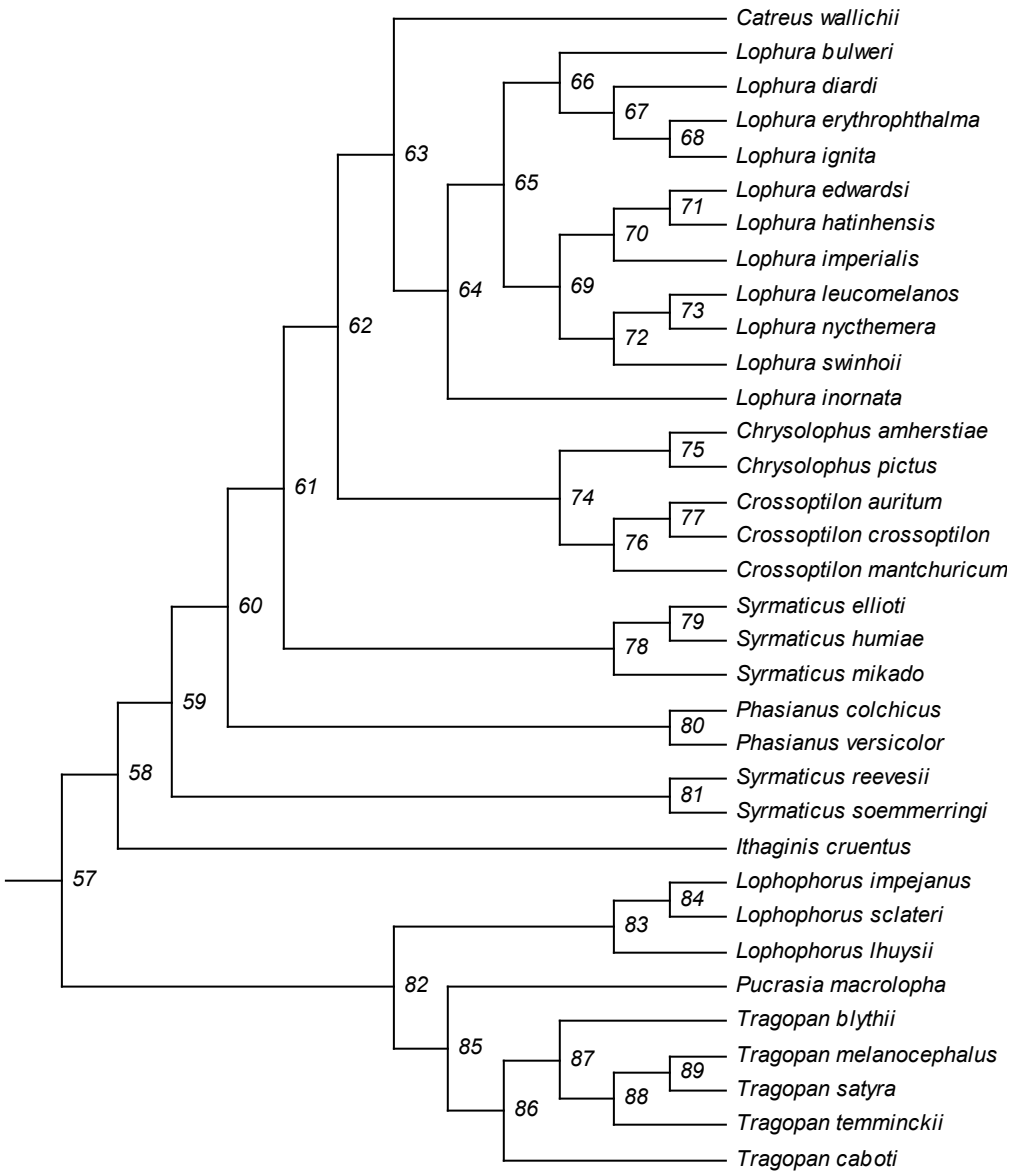

Figure 2.3: continued: N) Phasianinae II.

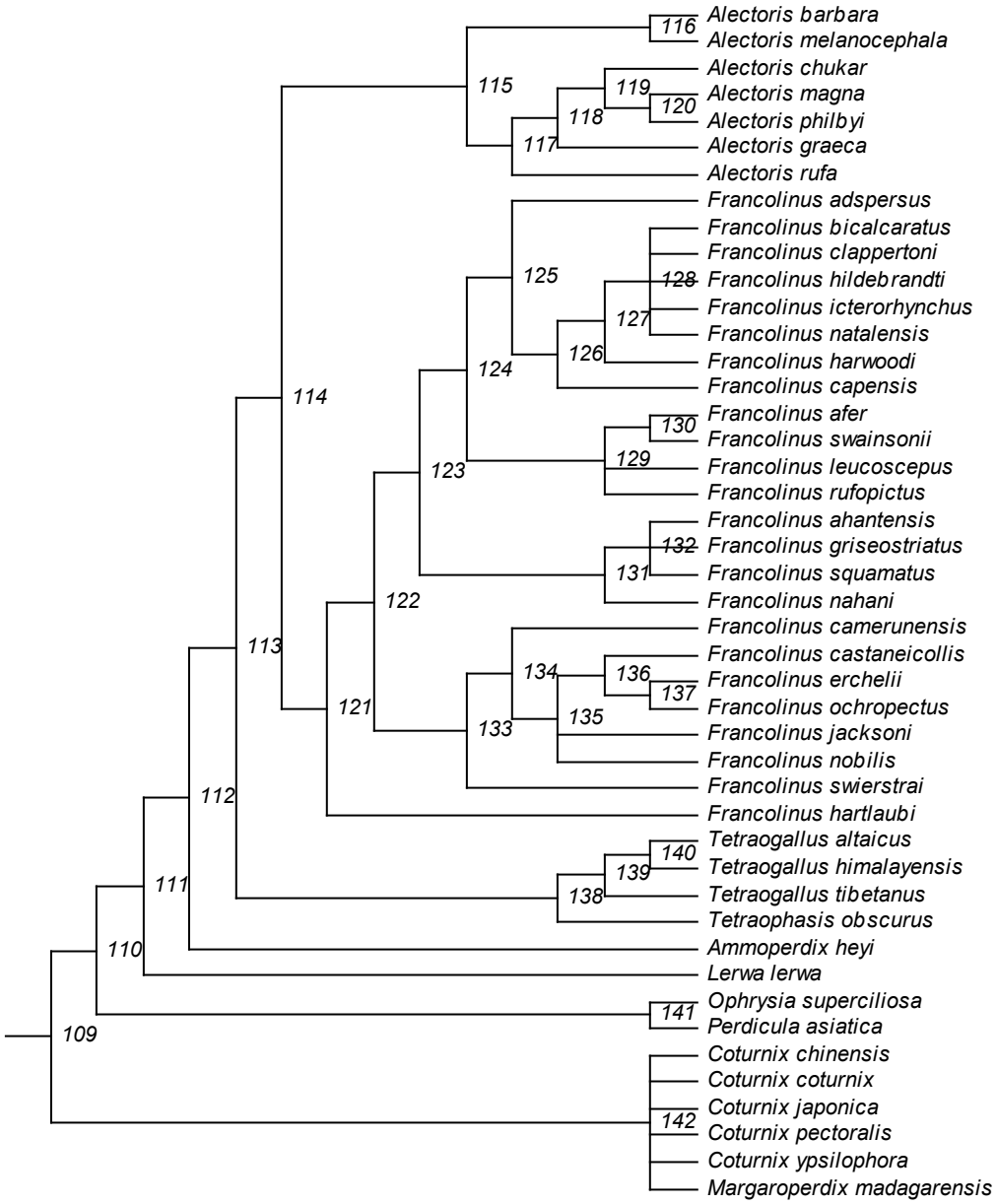

Figure 2.3: continued: O) Perdicinae I.

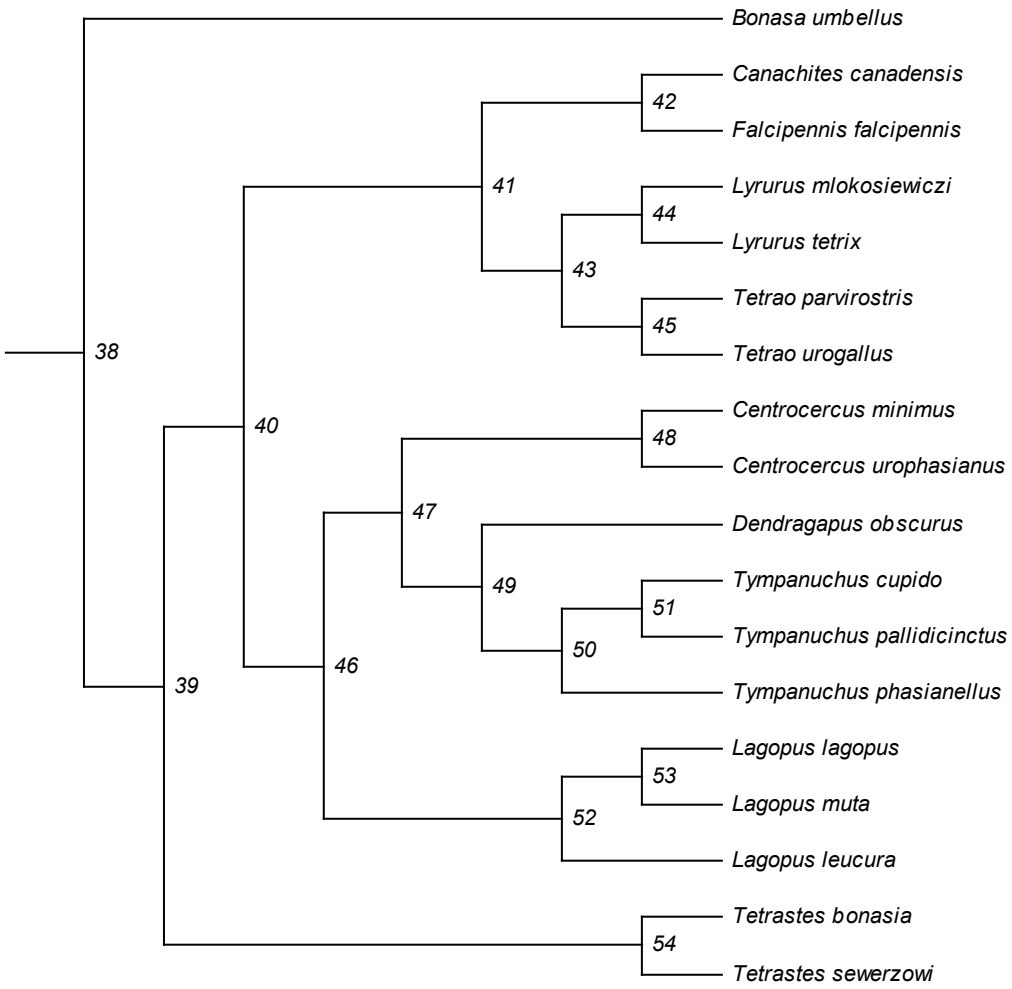

Figure 2.3: continued: P) Tetraoninae.

CHAPTER 3

SUBSPECIES AND UNITS FOR CONSERVATION AND MANAGEMENT OF THE NORTHERN BOBWHITE IN THE EASTERN UNITED STATES[1]

[1] Eo, S. H., Wares, J. P., and Carroll, J. P. To be submitted to *Conservation Genetics*

ABSTRACT

The northern bobwhite (*Colinus virginianus*) is a small game bird with sedentary lifestyles and has experienced population decline throughout most of its native distribution in the eastern United States. We investigated intraspecific genetic relationships among 14 local populations covering four putative subspecies (*C. v. marilandicus*, *C. v. virginianus*, *C. v. mexicanus*, and *C. v. floridanus*) in the United States. Analysis of mitochondrial DNA sequences revealed that there is small, but significant, genetic structure of northern bobwhite populations or subspecies in the eastern U.S. However, our results did not support current subspecies limits as distinct evolutionarily significant units, based on the amount of population genetic divergences and insufficient lineage sorting of mtDNA haplotypes among subspecies. Instead, our results suggest that *C. v. virginianus*, *C. v. marilandicus*, and *C. v. mexicanus* be merged into a single management unit, and *C. v. floridanus* be considered as another distinct unit for conservation and management.

INTRODUCTION

The northern bobwhite (*Colinus virginianus*) is a small game bird found in shrubs or forest-edge habitats, has a sedentary lifestyle, and is distributed from the eastern United States to Mexico (Johnsgard 1988; Carroll 1994; Brennan 1999). The species has experienced population declines throughout most of its native geographic distribution due in large part to habitat loss and fragmentation during the past >40 years (Burger 2002). Despite its relative abundance as a popular game species, the northern bobwhite was recently listed as "Near Threatened" by the IUCN (IUCN 2006). For game management and conservation purposes, many introductions and translocations of the northern bobwhite have been carried out in the United States (see references

in Scott 1985). However, the effects of introductions and translocation programs have been mostly unknown or unsatisfactory, in part because these efforts often have not considered historical population structure, subspecies ranges, or any genetic information (Scott 1985; Roseberry et al. 1987; Brennan 1999). Although genetic information may offer a way of conservation and management of species (Avise 2000; Zink et al. 2000; Frankham et al. 2002), few genetic studies have been attempted to delineate subspecies ranges or to identify distinct populations in northern bobwhites (but see Ellsworth et al. 1989; Nedbal et al. 1997).

As many as 22 subspecies of the northern bobwhite (up to seven of which are found in the United States) have been recognized using male plumage variation across geographic ranges as a criterion (Holman 1961; Johnsgard 1988; Carroll 1994; Brennan 1999; Dickinson 2003). Traditionally, subspecies have served as a unit for classification and/or evolutionary theories, but recently they have been used as accepted units for conservation or management of vertebrate species (Ryder 1986; Avise 2000; Crandall et al. 2000; Zink 2004). Particularly, for conservation biologists and wildlife managers, a matter of interest is whether a species is demographically connected across its geographic range, or is divisible into subunits due to the distribution of genetic diversity or demographic structure. If a subspecies has a long history of evolving independently, a mitochondrial DNA (mtDNA) gene tree may show a pattern of reciprocal monophyly (Avise 2000). In this respect, taxonomic category of subspecies may serve as a surrogate for evolutionarily significant units (ESUs, Ryder 1986; Moritz 1994) and play a central role for evolution and conservation of the taxa. Subspecies or populations that do not show a pattern of reciprocal monophyly in mtDNA gene tree, but that are significantly diverged in allele frequencies at neutral loci, are still important for conservation as management

units (MUs, Moritz 1994; Avise 2000). Such populations may be connected by low levels of gene flow, thereby representing functionally independent populations (Moritz 1994).

The identification of distinct genetic units is an important step for the management of natural populations as well as taxonomic delineations within a given taxon (Ryder 1986; Moritz 1994; Avise 2000; Crandall et al. 2000; Fraser and Bernatchez 2001; Frankham et al. 2002; Zink 2004; Elser et al. 2006; Palsboll et al. 2007). However, despite being one of the most studied birds in the world (Chumchal 2008), it is unknown if current morphology-based subspecific delineations of northern bobwhite reflect these units. Here, we investigated levels of genetic differentiation of populations within and among subspecies of the northern bobwhite in the Eastern United States, using the mitochondrial DNA control region (mtDNA CR). Rapidly evolving mtDNA CR may show good resolution of intraspecific structure of a phylogenetic tree and evidence of lineage sorting in relation to ecological variation (Barrowclough et al. 2004; Russell et al. 2005). Our aims were to assess levels of genetic diversity and population structure within and among subspecies of the northern bobwhites using mtDNA CR, and to test if the current subspecies designations are supported by the phylogenetic and statistical structure of mtDNA sequences.

MATERIALS AND METHODS

During 2000-2006, we collected wings or feather samples from 153 hunter-killed northern bobwhites, representing 14 local populations in 12 states (FL, GA, IL, IN, KY, MS, NC, NJ, NY, SC, TN, and VA) within the distribution of 4 putative subspecies (*C. v. marilandicus*, *C. v. virginianus*, *C. v. mexicanus*, and *C. v. floridanus*) across the Eastern United States (Figure 3.1). Before collecting samples, we consulted state natural resources agencies managing this species

in order to ensure our samples came from wild and native populations, rather than from managed historical introductions and translocations of the northern bobwhite throughout its geographic range, which could complicate recovered genetic patterns.

Genomic DNA of northern bobwhites was extracted from muscle tissues using the DNeasy Tissue Kit (Qiagen). For the polymerase chain reaction (PCR) amplification of mtDNA CR, the forward primer GLU3 (5'-GSTTGAAAARCCATYGTTGTTCTCAACTACG-3') and the reverse primer PHE2 (5'-TRNRTACCRTCTTGGCATCTTCAGTGC-3') were designed. All PCR amplifications were conducted in 20 μL reactions containing 1× PCR buffer, 2.5 mM MgCl2, 200 μM dNTPs, 0.5 μM primers, 1 U of *Taq* DNA polymerase (AmpliTaq Gold®), and 40 ~ 100 ng DNA, using the following program: one cycle of 3 min at 95°C, 40 cycles of denaturation at 95°C for 30s, primer annealing at 58°C for 30s, and elongation at 72°C for 1 min. A final elongation step at 72°C for 10 min was added followed by cooling to 4°C. PCR products were purified with Exosap-IT (Amersham Biosciences) and were sequenced in an automated DNA sequencer (ABI 3730) using the BigDye 3.1 terminator cycle-sequencing kit (Applied Biosystems) with the following conditions: one cycle of 1 min at 96°C, and 99 cycles of 96°C for 10s, 50°C for 5s, and 60°C for 4 min. After amplification and sequencing of the whole mtDNA CR using both primers GLU3 and PHE2, a new primer H614 (Sorenson et al. 1999) was additionally used to better sequence the left half of the sequences (5' end side of the mtDNA CR). Sequences were compiled in the program Sequencher v.4.5 (Gene Codes), and low quality sequence regions as determined using Phred scores (Ewing et al. 1998) were not analyzed. Sequences were aligned in the program ClustalX (Thompson et al. 1997) with default conditions and edited manually. Sites with gaps were not considered for analysis.

Basic diversity parameters such as haplotype diversity (h) and nucleotide diversity (π) were calculated for each local population or subspecies using the program ARLEQUIN v.3.11 (Excoffier et al. 2005). A median-joining network analysis was performed to explore the relationships of the northern bobwhite mtDNA CR haplotypes, using the program NETWORK v.4.5 (http://www.fluxus-technology.com). Networks can better depict relationships among the sampled haplotypes at the intraspecific level than phylogenetic methods because networks allow for concurrent existence of extant ancestral and descendant haplotypes (Bandelt et al. 1999; Posada and Crandall 2001).

We tested the hypothesis of random distribution of the individuals between pairs of local populations and subspecies based on haplotype frequencies (Raymond and Rousset 1995; Goudet et al. 1996). A Markov chain set to 100,000 steps with run of 10,000 of dememorization was used to obtain an unbiased estimate of exact probabilities. Also, to investigate patterns of genetic structure among sampled populations in relation to geographic distribution, we conducted the Mantel test, using the web-based program IBDWS v.3.15 (Jensen et al. 2005). Levels of differentiation among local populations or subspecies were assessed using pairwise F_{ST} and hierarchical analyses of molecular variance (AMOVA, Excoffier et al. 1992). A distance matrix with the Tamura and Nei (1993) model was used for pairwise F_{ST} and AMOVA because this model is appropriate to describe the evolution of mtDNA CR sequences. We based a priori groupings for AMOVA on several scenarios to distinguish whether differences among populations or subspecies are better explained by current subspecies limits or geographical proximity. Each procedure for pairwise F_{ST} and AMOVA was repeated for 10,000 random permutations to assess significance.

To detect the trends in population size, we used the frequency distribution of the number of pairwise nucleotide differences among individuals (the mismatch distribution) using a generalized least-squares approach (Excoffier et al. 2005). This distribution is expected to be multimodal for populations at demographic equilibrium (Rogers and Harpending 1992), and to be unimodal for populations having passed through a recent demographic expansion (Rogers and Harpending 1992) or through a range expansion with high levels of migration between adjoining populations (Ray et al. 2003). An expected distribution under the sudden demographic expansion model was generated using a thousand parametric bootstrap replicates, and compared with the observed frequency distribution using ARLEQUIN v.3.11 (Excoffier et al. 2005). In addition, we employed Tajima's *D* test to determine whether the sequences conformed to neutral equilibrium expectations (Tajima 1989). The presence of significant departures from the null hypotheses may suggest either changes in population size or selective pressures on the sequence, both of which are expected to generate negative *D* values. In contrast, processes such as population subdivision, balancing selection or recent population bottlenecks are expected to take positive *D* values (Simonsen et al. 1995; Fay and Wu 1999; Ramos-Onsins and Rozas 2002). We further applied Fu's F_S test of neutrality (Fu 1997), which may lead to negative F_S values in expanded populations and it is considered as one of the strongest tests in detecting traces of population expansions (Ramos-Onsins and Rozas 2002).

RESULTS

The mtDNA CR sequence alignment (655 bps) from 153 northern bobwhites showed 41 different haplotypes, defined by 25 polymorphic sites. Haplotype diversity was high with overall $h = 0.89 \pm 0.018$ across all individuals, but both nucleotide diversity and mean pairwise

differences between haplotypes were low within all subspecies (overall $\pi = 0.34 \pm 0.21\%$; $k = 2.202 \pm 1.224$; Table 3.1). A median-joining network revealed no clear pattern of structure among haplotypes or subspecies (Figure 3.2). The most common haplotype was found in 43 individuals (28.1% of all samples). This haplotype was dominant in populations representing 3 putative subspecies (*C. v. marilandicus*, *C. v. virginianus*, and *C. v. mexicanus*), but was not identified in *C. v. floridanus*. The second most common haplotype with frequency of 11.1% was detected in all 4 subspecies. A haplotype, which was observed in more than the half of *C. v. floridanus* individuals, was not found in other subspecies except for a single individual in *C. v. mexicanus*. The frequency of novel haplotypes in each subspecies ranged from 9.1% (*C. v. marilandicus*) to 33.8% (*C. v. mexicanus*).

Genetic differentiation across both sampled subspecies and populations based on haplotype frequency distribution over all samples was highly significant ($P < 0.0001$; 30,000 Markov chain steps), suggesting globally structured relationships among samples. A pattern of genetic structure across sampled populations in relation to geographic distribution demonstrated a weak, but significant, correlation, explaining 14% of variance (one-tailed $P = 0.0056$; 30,000 randomizations; Figure 3.3). However, the Mantel tests within *C. v. mexicanus* and within the group including *C. v. marilandicus* and *C. v. virginianus* produced non-significant correlations, and did not support relationships of isolation by distance (one-tailed $P > 0.5$; not analyzed within the other subspecies due to small sample size). Pairwise F_{ST} and exact tests of differentiation between subspecies showed that they were significantly differentiated from one another ($P < 0.05$; Table 3.2); however, *C. v. marilandicus* and *C. v. virginianus* were not different. At the population level (with more than 5 individuals), many comparisons were not significant with low value of F_{ST} ($P > 0.05$ in 49 of 66 cases; average $F_{ST} = 0.0771$), whereas 10 of 11 comparisons

between the *C. v. floridanus* population and the other subspecies populations were significant with high value of F_{ST} ($P < 0.05$; average $F_{ST} = 0.1914$). Analysis of molecular variance showed that 94.3% of total genetic variance was explained by the variations of individuals within populations and only 4.7% by the variation among putative subspecies, suggesting weak signal of genetic structuring at the subspecies level (Table 3.3). Variance among subspecies slightly increased up to 6.1% when *C. v. marilandicus* and *C. v. virginianus* were considered as one group (Table 3.3). When *C. v. floridanus* was omitted from the analysis, variance among the remaining subspecies was not significant (Table 3.3).

The mismatch distribution analysis rejected the population expansion model when applied to the all samples ($P = 0.02$; Figure 3.4). However, pooling differentiated samples or different subspecies may produce some biases (Rajabi-Maham et al. 2008). When we conducted the analysis subspecies by subspecies, all within-subspecies analyses, including a merged group of *C. v. marilandicus* and *C. v. virginianus*, showed unimodal distributions, except *C. v. floridanus*, suggesting that they conformed to the model of sudden expansion. The distribution for *C. v. floridanus* does not seem to be unimodal, but this may need to be re-analyzed with more sample collections. Tajima's *D* and Fu's F_S were negative values in the majority of northern bobwhite populations or subspecies (Table 3.1). Tajima's *D*s were not significantly different from zero for all individual populations or subspecies, but it was significantly negative for pooled samples. Fu's F_S were significant, large negative values for pooled samples, *C. v. virginianus*, and *C. v. mexicanus*.

DISCUSSION

Several lines of evidence revealed small, but significant, levels of genetic differentiation in the northern bobwhites: global genetic structure of the northern bobwhites, significant F_{ST}-values among subspecies, and restricted gene flow via isolation by distance across the subspecies ranges. The geographic limits of northern bobwhite subspecies in the study area are generally associated with major barriers, including the Appalachian Mountains (dividing *C. v. virginianus* and *C. v. marilandicus* from others), and also possibly with the peninsular effect dividing populations from Florida and the mainland (dividing *C. v. floridanus* from others). A diverse array of co-distributed taxa on one side divided by those barriers has shown morphological, ecological, behavioral, and other life-history distinctions from their relatives on the other side (Remington 1968; Avise 2000; Soltis et al. 2006). This phenomenon was verified in northern bobwhites, characterized by weak, but significant, genetic differentiations along some subspecies lines.

However, it may be much more informative for conservation and management purposes to delineate subspecies or subunits based on the amount of population genetic divergence instead of simply the rejection of panmixia (Palsboll et al. 2007). Levels of mtDNA variability did not support current subspecific status of any of the four sampled subspecies in the Eastern United States. Despite overall significant genetic structuring, the levels of genetic divergence among subspecies or among populations were quite low. Less than 5% of total genetic variance was explained among subspecies, and *C. v. virginianus* and *C. v. marilandicus* were not differentiated. Whatever structuring we observed was mainly due to *C. v. floridanus*. The mtDNA haplotypes did not show reciprocally monophyletic structure for any single subspecies. Also, weak but significant patterns of isolation by distance across the range suggest that northern bobwhites in this area have arrived at genetic equilibrium between migration and drift. We, therefore, suggest

that there is little evidence of multiple ESUs or distinct subspecies in our sampling area although the species is considered highly sedentary with restricted dispersal rates (Johnsgard 1988; Carroll 1994; Brennan 1999).

Inconsistency between current subspecies and our molecular data reveals that intraspecific taxonomy of the northern bobwhite has been poorly studied and largely unknown. For example, many authors use independent subspecies limits for *C. v. marilandicus* and *C. v. virginianus* (Johnsgard 1988; Carroll 1994; Brennan 1999), whereas some consider *C. v. marilandicus* as synonym for *C. v. virginianus* (Dickinson 2003). This discrepancy may be because classifications have been based mainly on plumage characteristics and often from just a few specimens (Johnsgard 1988). Inconsistency between subspecies taxonomies based on morphology and molecular data has been observed in other Galliformes birds, for example, in some subspecies of the wild turkey *Meleagris gallopavo* (Mock et al. 2002), sage grouse *Centrocercus urophasianus* (Benedict et al. 2003), capercaillie *Tetrao urogallus* (Liukkonen-Anttila et al. 2004), and sharp-tailed grouse *Tympanuchus phasianellus* (Spaulding et al. 2006). These studies generally suggest that the recent divergence of designated subspecies in many species may reflect contemporary population fragmentation, present-day gene flow, and/or some local adaptations.

The lack of support in mtDNA data for the morphological taxonomy is common in avian species (Ball and Avise 1992; Avise and Walker 1998; Zink et al. 2000; Zink 2004). A phylogeographic survey of 41 avian species based on mtDNA revealed an average number of ESUs or expected subspecies of 1.9 whereas the average number of designated subspecies was 5.5 (Zink 2004). Our results for the northern bobwhite based on sampling of the four designated subspecies also suggested only one expected subspecies. This unbalanced phenomenon is likely

because subspecies have been often based on arbitrarily single morphological characters that are probably managed by relatively few genes and affected individually by different selective pressures (Zink et al. 2000). In contrast, genetic characters, such as mtDNA genes, may represent overall demographic factors and population history (Zink et al. 2000). However, it is also possible that morphological variation could be consistent with distinct subspecies along the geographical structure regardless of neutral genetic variation. Such adaptive traits may not follow neutral variation patterns, and evolution of such traits can be more rapid than that of complete lineage sorting in the single-locus mtDNA genome (Crandall et al. 2000; Fraser and Bernatchez 2001; Palkovacs et al. 2004; Palsboll et al. 2007). Therefore, it is important to appropriately define subspecies or population groups which may serve as proxies of evolutionarily independent lineages or units for conservation when we need to delineate distinct units to effectively manage the species or population trends (Moritz 1994; Moritz et al. 1995; Avise 2000; Crandall et al. 2000; Zink et al. 2000; Zink 2004; Palsboll et al. 2007).

The lack of a clear mtDNA pattern among the four subspecies could reflect the impact of a history of widespread translocations (Scott 1985). However, we tried to minimize the sampling of potentially nonnative birds. Assuming no translocated sampling in this study, degrees of differentiation may have resulted from recent widespread colonization processes, with some common haplotypes having evolved before the current population structure had been formed (Bulgin et al. 2003). The trend for recent expansion and colonization processes was reflected by the mismatch distribution, and negative values of both Tajima's D and Fu's F_S. In the 18th and 19th centuries, the northern bobwhites rapidly extended its range into the Midwest where formerly the vast expanses of grass severely limited its usefulness for the species, with deforestation and cultivation of northeastern United States by European settlers (Forbush 1912;

Edminster 1954; Bent 1963). Their recent colonization processes and current widespread distribution suggest they have not diverged enough to reach a state of reciprocal monophyly.

CONSERVATION IMPLICATIONS

Although *C. v. floridanus* did not show monophyly or complete mtDNA haplotypes sorting as an ESU, significant pairwise F_{ST}-values and AMOVA results showed that the subspecies exhibited different genetic structures from the others. Based on genetic structure and geographic ranges reflecting historical population process, our analyses suggest that *C. v. floridanus* should be considered as a distinct unit for conservation or management (Moritz 1994; Moritz et al. 1995; Palsboll et al. 2007). In contrast, *C. v. virginianus*, *C. v. marilandicus*, and *C. v. mexicanus* should be considered a single management unit because levels of genetic divergence among these putative subspecies were quite low. However, genetic information reflects patterns of both historical and contemporary issues. As such, from the genetic data only, one cannot always infer population processes or dynamics at time frames measured in years to decades, which would be primary issues for the majority of wildlife management (Crandall et al. 2000; Elser et al. 2006). To clarify this subspecies as an obvious management unit for the conservation purpose, it is highly recommended that ecological studies with morphological, demographic, and behavioral information, as well as genetic relationships should be undertaken and interpreted (Crandall et al. 2000).

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Table 3.1: Northern Bobwhite (*Colinus virginianus*) collection sites (mapped in Figure 3.1), genetic polymorphism, and hypotheses of demographic history for different subspecies or populations, based on mtDNA CR sequences.

			Genetic polymorphism			Demographic history	
Subspecies / populations	*N(I)*	*N(H)*	*h* ± *SD*	π ± *SD* (%)	*k* ± *SD*	Fu's *Fs*	Tajima's *D*
C. v. marilandicus	33	10	0.860 ± 0.037	0.33 ± 0.21	2.189 ± 1.242	-2.52	-0.85
New York (NY)	7	4	0.857 ± 0.102	0.44 ± 0.30	2.876 ± 1.715	0.56	0.85
New Jersey (NJ)	10	3	0.622 ± 0.138	0.11 ± 0.10	0.713 ± 0.582	-0.16	0.02
Virginia (VA)	16	7	0.850 ± 0.060	0.40 ± 0.25	2.637 ± 1.485	-0.92	-0.80
C. v. virginianus	43	16	0.880 ± 0.032	0.32 ± 0.20	2.066 ± 1.180	-9.08 **	-1.28
North Carolina (NC)	28	12	0.897 ± 0.031	0.34 ± 0.22	2.250 ± 1.276	-5.13 **	-0.91
South Carolina (SC)	4	4	1.000 ± 0.177	0.46 ± 0.36	3.021 ± 1.975	-1.24	-0.81
Florida-Georgia (FLGA)	11	6	0.727 ± 0.144	0.20 ± 0.15	1.315 ± 0.755	-2.74 **	-0.89
C. v. mexicanus	68	28	0.882 ± 0.031	0.34 ± 0.21	2.230 ± 1.244	-25.31 **	-1.34
Illinois (IL)	10	4	0.644 ± 0.152	0.15 ± 0.12	0.959 ± 0.712	-0.97	-1.24
Indiana (IN)	19	10	0.912 ± 0.040	0.34 ± 0.22	2.247 ± 1.293	-4.29 **	-0.78
eastern Kentucky (KYe)	2	2	1.000 ± 0.500	0.31 ± 0.38	2.007 ± 1.737	0.69	0.00
middle Kentucky (KYm)	6	6	1.000 ± 0.096	0.48 ± 0.33	3.154 ± 1.895	-3.18 **	-0.62
Kentucky-Tennessee (KYTN)	11	9	0.946 ± 0.066	0.40 ± 0.26	2.597 ± 1.504	-5.29 **	-0.23
Mississippi-Tennessee (MSTN)	14	10	0.923 ± 0.060	0.34 ± 0.23	2.244 ± 1.313	-6.05 **	-1.13
eastern Tennessee (TNe)	6	4	0.867 ± 0.129	0.44 ± 0.31	2.893 ± 1.762	0.15	-1.07
C. v. floridanus	9	4	0.694 ± 0.147	0.24 ± 0.18	1.562 ± 1.025	-0.13	-0.65
Florida (FL)	9	4	0.694 ± 0.147	0.24 ± 0.18	1.562 ± 1.025	-0.13	-0.65
Total	153	41	0.889 ± 0.018	0.34 ± 0.21	2.202 ± 1.224	-27.11 **	-1.44 *

N(I), number of individuals; *N(H)*, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity (%); *k*, mean pairwise differences; *SD*, standard deviation; **, significant at $P < 0.01$; *, significant at $P < 0.05$.

Table 3.2: Pairwise F_{ST} (below diagonal) and the significance of Exact tests of differentiation (above diagonal) among the northern bobwhite subspecies in the study area, based on mtDNA control region.

	C. v. marilandicus	*C. v. virginianus*	*C. v. floridanus*	*C. v. mexicanus*
C. v. marilandicus			$^{+++}$	$^{+}$
C. v. virginianus	-0.0053		$^{+++}$	$^{+}$
C. v. floridanus	0.1888 ***	0.1522 **		$^{++}$
C. v. mexicanus	0.0455 **	0.0246 *	0.1527 ***	

* or $^{+}$ $P < 0.05$, ** or $^{++}$ $P < 0.01$, and *** or $^{+++}$ $P < 0.001$, with 10,000 random permutations for pairwise F_{ST}, or a Markov chain of 100,000 steps for the Exact tests of differentiation.

Table 3.3: Analysis of molecular variance in the northern bobwhites with several *a priori* grouping scenarios, based on putative subspecies.

Grouping	Source of variation	df	% variation	
All 4 subspecies separated	Among subspecies	3	4.68	**
	Among populations within subspecies	10	1.03	
	Within populations	139	94.29	**
(*C. v. marilandicus* - *C. v. virginianus*) combined	Among subspecies	2	6.14	**
	Among populations within subspecies	11	0.71	*
	Within populations	139	93.15	**
All 4 subspecies combined	Among subspecies	.	.	
	Among populations within subspecies	13	4.57	**
	Within populations	139	95.43	
All subspecies separated, without *C. v. floridanus* in analysis	Among subspecies	2	2.31	
	Among populations within subspecies	10	0.88	
	Within populations	131	96.81	*

* $P < 0.05$ and ** $P < 0.01$.

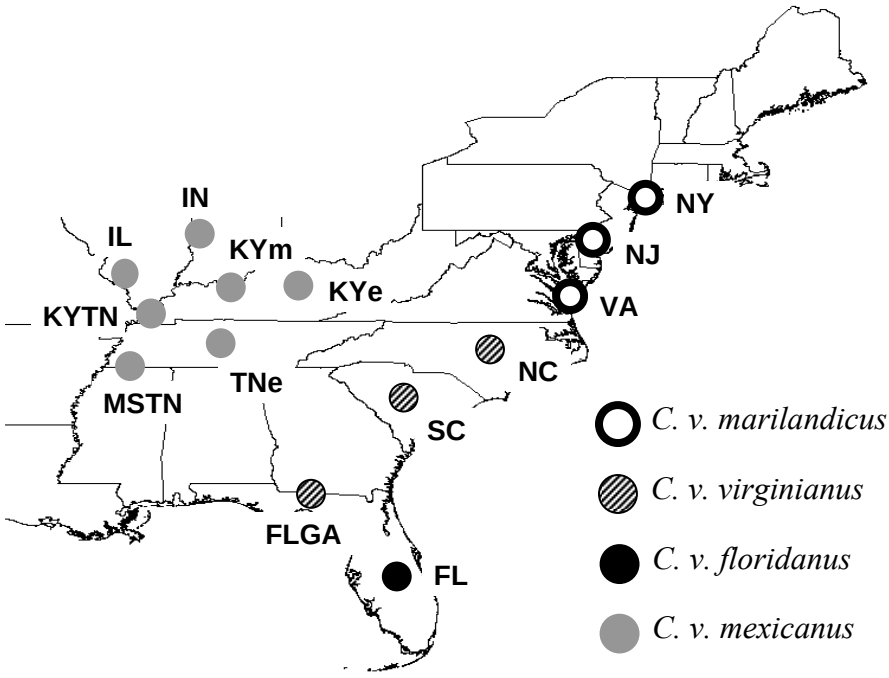

Figure 3.1: Sample populations and putative subspecies of the northern bobwhites in this study. See the Table 3.1 for population acronyms and sample sizes.

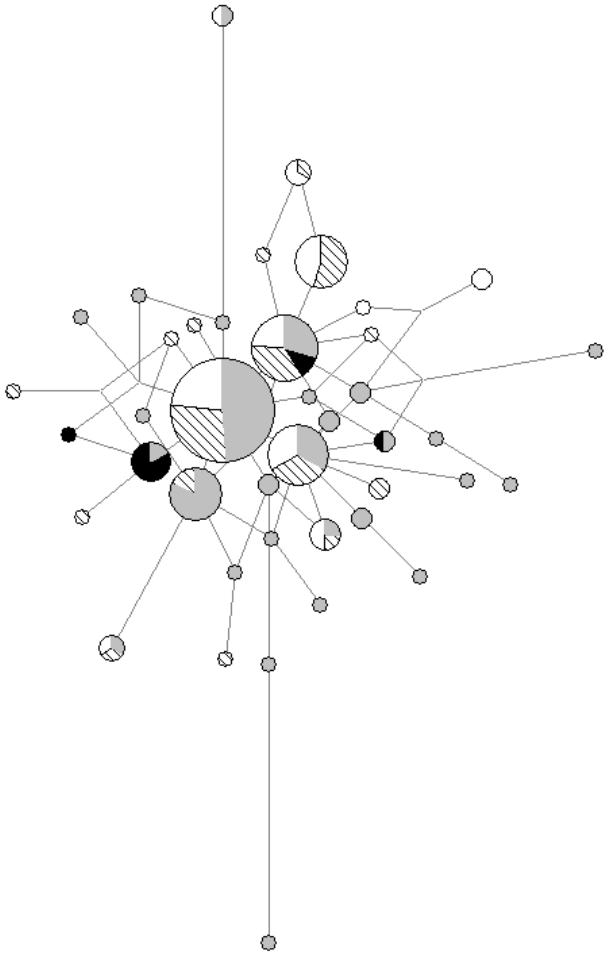

Figure 3.2: A Median-joining network for 41 haplotypes of the northern bobwhites. The relative sizes of the circles represent the number of individuals contained within each haplotype, and the pie slices represent the portion of each subspecies (open, *C. v. marilandicus*; hatched, *C. v. virginianus*; black, *C. v. floridanus*; gray, *C. v. mexicanus*).

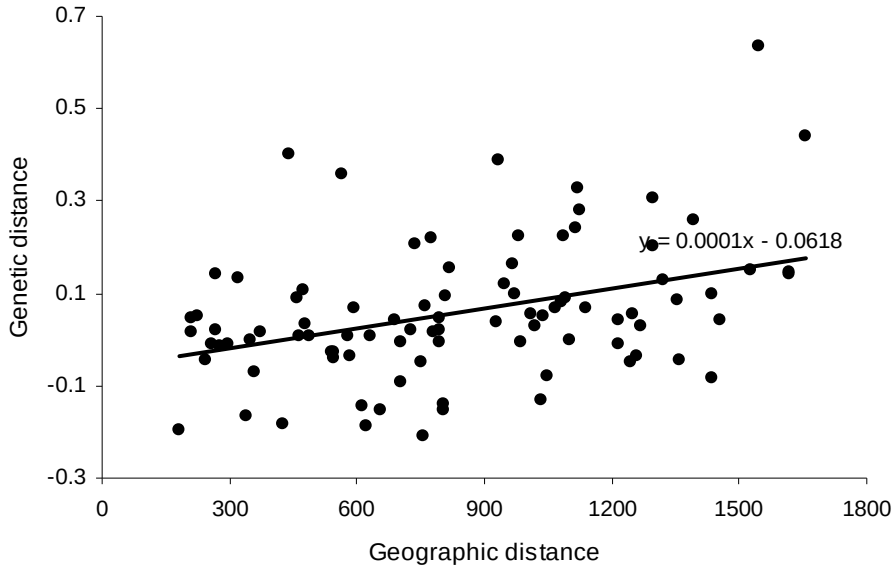

Figure 3.3: Genetic distance, $F_{ST}/(1-F_{ST})$, based on mtDNA control region versus geographic distances, km, for all pairwise combinations of 14 northern bobwhite populations. A significant positive correlation was observed ($y = 0.0001x - 0.062$, $r = 0.37$, $P = 0.0056$, 30 000 randomizations, Mantel test).

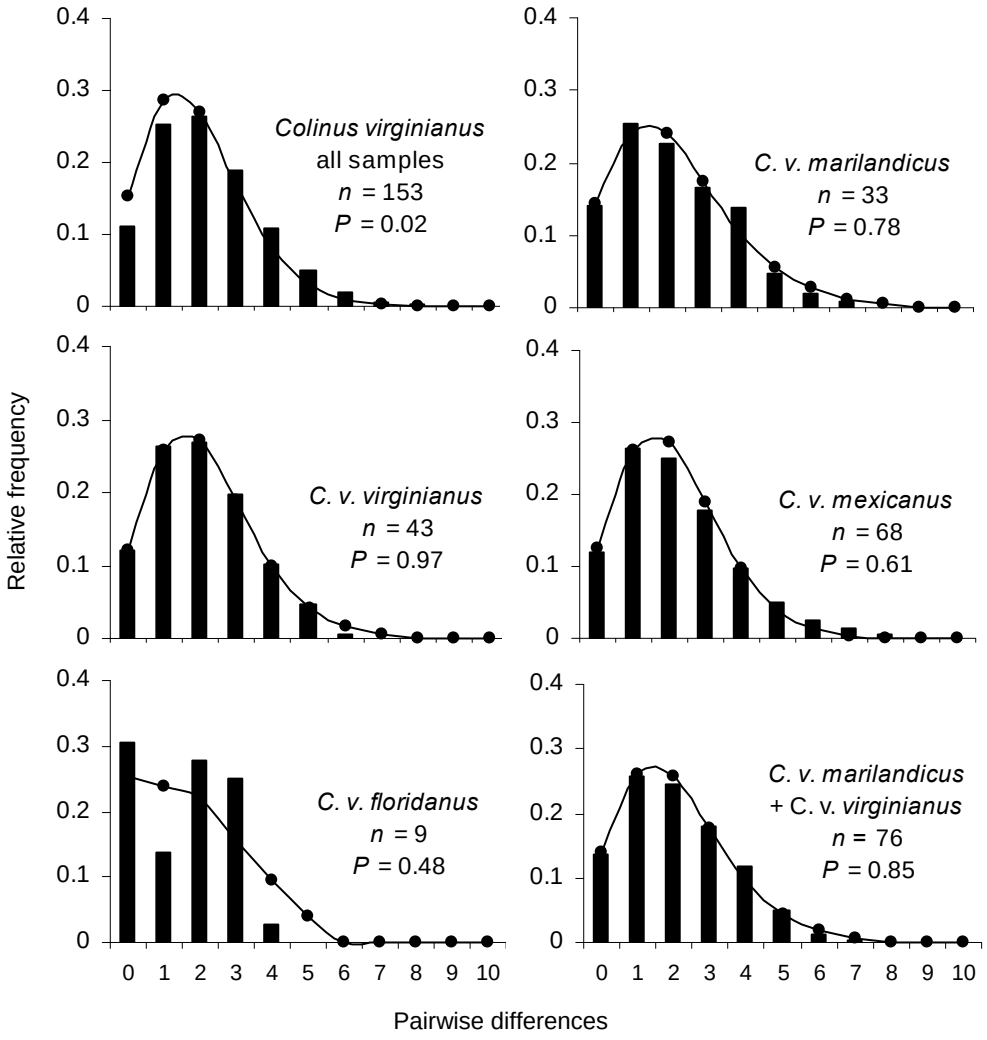

Figure 3.4: Mismatch distributions for all pooled samples and four putative subspecies of the northern bobwhites. The observed distributions (bars) were compared with expected distributions under a model of sudden expansion (black circles and solid lines). *P* values were calculated as the proportion of simulations producing a larger sum-of squared deviation (SSD) than the observed SSD.

CHAPTER 4

EXTREMELY HIGH OR TOO LOW GENETIC DIFFERENTIATION IN HIGHLY POLYTYPIC WIDESPREAD NORTHERN BOBWHITES: CONGRUENT OR CONFLICTING PATTERNS IN MITOCHONDRIAL AND NUCLEAR MICROSATELLITE LOCI[1]

[1] Eo, S. H., Wares, J. P., Faircloth, B. C., Terhune, T. M., and Carroll, J. P. To be submitted to *Molecular Ecology*

ABSTRACT

Genetic information of species in nature can provide not only insights into population structure and subspecies relationships, but also implications for conservation and management of the species. Here, we examine the patterns of genetic variability in the northern bobwhite (*Colinus virginianus*) across the species' range in the USA, using mitochondrial and nuclear microsatellite loci. Congruent results from both mitochondrial and microsatellite markers revealed that extremely high genetic differentiation between isolated Arizona northern bobwhites (masked bobwhite, *C. v. ridgwayi*) and the other widespread populations in midwestern and eastern USA. Compared to the Arizona population, in contrast, the others across six subspecies were not substantially different from each other, showing too low genetic differentiation, with some possible exceptions. Also, the masked bobwhite showed the lowest genetic diversity among all subspecies and populations analyzed. This genetic information reflects that the masked bobwhite have experienced a severe range contraction and decline in population size and therefore why the subspecies has been listed as endangered and faced by local extinction. It is highly recommended to assign more conservation priority and effort to the masked bobwhite as a both morphologically and genetically distinct subspecies. Although both markers congruently indicated overall genetic patterns, we found that the genetic differentiation among population (e.g., F_{ST} or R_{ST}) was much apparent at mitochondrial DNA marker. Such conflicting patterns can be explained by differences of mutation process and rate for both markers. Also, male-biased gene flow across the species range may explain for the increased genetic divergence observed in mitochondrial DNA relative to microsatellite loci.

INTRODUCTION

Genetic characteristics in natural populations are influenced by demographic, environmental and genetic processes such as gene flow, genetic drift, and natural selection as well as geographic and landscape features of habitats (Avise 2000; DeSalle & Amato 2004). For species living low elevation with sedentary life style, landscape features such as high mountain ranges may isolate their habitats and restrict dispersal of individuals among habitats. In such populations, restricted gene flow and potentials of genetic drift and adaptive divergence may promote population differentiation (Whitlock & Barton 1997; Gibbs 2001; Templeton *et al.* 2001; Brooker & Brooker 2002). In contrast, it may be difficult to delineate completely distinct populations in continuous habitats where no physical barriers exist though individuals can be divided into subpopulations connected by variable rates of gene flow, forming a pattern of isolation by distance (Wright 1943; Slatkin 1993; Rousset 1997, 2000; Berthier *et al.* 2005). Genetic characteristics of populations are influenced by different degrees of which adjacent populations are spatially isolated and vary in size (MacArthur & Wilson 1967; Eckert *et al.* 2008). Even for species with widespread continuous habitats, spatially separated small-size populations existing near their marginal ranges can exhibit genetically distinct feature. Smaller and more isolated populations may be at high risk of extinction because effective population size and rate of gene flow are expected to be lower at such populations than at the central populations of the range.

The northern bobwhite *Colinus virginianus* is the most widely distributed New World quail. The species inhabits the continent east of the Rocky Mountains and the Sierra Madre Occidental in North America. The range of the species extends north to Ontario, Canada, southeast to the Florida peninsula and south to Mexico and adjacent countries (Johnsgard 1988; Carroll 1994; Brennan 1999). Also small, isolated populations are found in parts of southern

Arizona and Sonora, Mexico. Northern bobwhite is generally grassland-preferred and forest edge-adapted species, but birds in Arizona or Sonora (called masked bobwhite) are relatively more adapted to hot habitats and more xeric or desert environment (Johnsgard 1988; Carroll 1994; Brennan 1999; Hernandez *et al.* 2006). In spite of widespread range of the species, northern bobwhites are of conservation and management concern because the species has experienced population declines throughout most of its range due in large part to habitat loss and fragmentation (Burger 2002; IUCN 2007). In particular, masked bobwhite is listed under the Endangered Species Act of 1973 (USFWS 1995). Geographically peripheral or isolated small populations, such as masked bobwhites, are expected to exhibit lower genetic diversity and higher genetic differentiation than central populations (Eckert *et al.* 2008). In fact, masked bobwhite is restricted to one reintroduced population in Arizona and two known native populations in Sonora, Mexico, with populations of only 1000 – 2000 individuals (Carroll 1994; Kuvlesky *et al.* 2000; Hernandez *et al.* 2006).

Of 22 named subspecies based largely on geographic location and morphology (Johnsgard 1988; Carroll 1994; Brennan 1999; Dickinson 2003), seven subspecies inhabit the USA (*C. v. marilandicus*, *C. v. virginianus*, *C. v. floridanus*, *C. v. mexicanus*, *C. v. taylori*, *C. v. texanus*, and *C. v. ridgwayi*, see Figure 4.1). However, this taxonomic classification is somewhat uncertain. It is based mainly on limited variation of male plumage in some subspecies, whereas females are almost indistinguishable (Brennan 1999). This uncertainty has been reflected in taxonomic history of this group. For example, Peters (1934) identified only four subspecies (*C. v. virginianus*, *C. v. floridanus*, *C. v. texanus*, and *C. v. ridgwayi*) in the USA, and considered *C. v. virginianus* encompassing all individuals across current ranges of *C. v. marilandicus*, *C. v. mexicanus*, and *C. v. taylori*. In contrast, masked bobwhite *C. v. ridgwayi* was identified as an

independent species *C. ridgwayi* from 1884 when the type specimen was collected until 1944 when it was reduced to subspecies status (Allen 1886; Aldrich 1946). The possibility that some subspecies are actually distinct species or just synonyms of other subspecies should be investigated more thoroughly with a diverse array of methods including both mitochondrial and nuclear genetic markers.

Identifying geographical boundaries and examining genetic structure of taxa can not only provide new insights about the evolutionary biology and ecology of the taxa but also be useful for their conservation and management implications (Ryder 1986; Moritz 1994; Avise 2000; Crandall *et al.* 2000; Fraser & Bernatchez 2001; Frankham *et al.* 2002; Zink 2004; Elser *et al.* 2006; Palsboll *et al.* 2007). Although a few genetic studies in northern bobwhite have been attempted they have focused on limited locations with relatively small sample size using a single marker system of either electrophoretic or mitochondrial data (Ellsworth *et al.* 1989; Nedbal *et al.* 1997; White *et al.* 2000). In Chapter 3, we delineated subspecies ranges and identified some genetically distinct units in northern bobwhites, but the study was restricted to eastern range of the species, based on only mitochodiral data. Here, we investigate genetic characteristics of natural populations of the northern bobwhite across large widespread and small isolated marginal populations, using both mitochondrial and nuclear microsatellite markers.

A number of studies have suggested that mitochondrial markers has the advantage of revealing significant genetic structure with geographic information (Zink 1997; Avise 2000; Barrowclough *et al.* 2004; Barrowclough *et al.* 2005), but it can be incongruent with nuclear markers due to, for example, the maternal inheritance mode of mtDNA, different rates of lineage sorting, and difference in effective population size between two markers (Crochet 2000; Funk & Omland 2003; Ballard & Whitlock 2004). The use of a new and independent genetic marker can

support or reject previously established hypotheses (Brito 2007). Congruent results from multiple loci can reduce the variance in the estimated parameters and conflicting patterns may also provide new insights that could not be revealed with a single loci alone (Prugnolle & de Meeus 2002; Brito 2007), For this reason, it is important to assess congruence or conflict among mitochondrial and nuclear loci for investigating genetic features in nature. In this study, we employed both mitochondrial control region sequences and 16 nuclear microsatellite loci to examine patterns and levels of genetic differentiation for the northern bobwhites populations, to describe possible factors responsible for shaping variable genetic structures across widespread or isolated marginal populations, and to explore whether genetic or demographic processes may have affected these results by comparing those from each marker system.

MATERIALS AND METHODS

Study Area and Sample Collection

We sampled and analyzed 560 northern bobwhites across 7 purported subspecies in the USA. We collected wings or feather samples from 525 hunter-killed northern bobwhites during hunting seasons of 2000 to 2006, representing 24 local populations in 18 states (Florida, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Mississippi, Missouri, Nebraska, New Jersey, New York, North Carolina, Oklahoma, South Carolina, Tennessee, Texas, and Virginia) within the distribution of 6 putative subspecies (*C. v. marilandicus*, *C. v. virginianus*, *C. v. floridanus*, *C. v. mexicanus*, *C. v. taylori*, and *C. v. texanus*) across the United States (Fig. 1). Additionally, feather samples from 35 masked bobwhites (*C. v. ridgwayi*) were collected from the Buenos Aires National Wildlife Refuge, Pima County, Arizona. A refuge masked bobwhite population was established at the Buenos Aires National Wildlife Refuge because it is one of the historic

habitats for the masked bobwhite and is adjacent to Sonora, Mexico, which currently contains the only remaining natural population. Our masked bobwhite samples were euthanized for lack of space at the refuge in 2008. Before collecting all samples in other populations, we consulted state natural resources agencies managing this species to ensure our samples came from wild and native populations, rather than from known historical introductions and translocations of the species which has occurred throughout its geographic range, which could complicate recovered genetic patterns. However, we cannot be absolutely certain that mixing of stocks among geographical regions has not occurred.

Laboratory Methods

Genomic DNA was extracted from muscle tissues or feathers using the DNeasy Tissue Kit (Qiagen). The entire mtDNA control region was successfully amplified by polymerase chain reaction (PCR) in 273 northern bobwhite samples, including 153 individuals from the previous study (chapter 3), using the forward primer GLU3 and the reverse primer PHE2 (chapter 3). All PCR amplifications were conducted in 20 μL reaction volumes containing 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM primers, 1 U of *Taq* DNA polymerase (AmpliTaq Gold®), and 40 ~ 100 ng of template DNA, using the following program: one cycle of 3 min at 95°C, 40 cycles of denaturation at 95°C for 30s, primer annealing at 58°C for 30s, and elongation at 72°C for 1 min. A final elongation step at 72°C for 10 min was added followed by cooling to 4°C. PCR products were purified with Exosap-IT (Amersham Biosciences) and were sequenced in an automated DNA sequencer (ABI 3730) using the BigDye 3.1 terminator cycle-sequencing kit (Applied Biosystems) with the following conditions: one cycle of 1 min at 96°C, and 99 cycles of 96°C for 10s, 50°C for 5s, and 60°C for 4 min. After amplification and sequencing of the

whole mtDNA CR using both primers GLU3 and PHE2, a new primer H614 (Sorenson *et al.* 1999) was additionally used to better sequence the left half of the sequences (5' end side of the mtDNA Control region). Sequences were compiled in SEQUENCHER v.4.5 (Gene Codes), and low quality sequence regions as determined using Phred scores (Ewing *et al.* 1998) were not analyzed. Sequences were aligned in CLUSTALX (Thompson *et al.* 1997) with default conditions and edited manually. Sites with gaps were not considered for analysis.

Microsatellite genotyping analysis for 513 northern bobwhites was conducted using 16 polymorphic primers: P1A7, P1F2, P1F3, P1H12, P2D7, PA12A, PA12G, PA1C, PA1F, PA3E, PA3F, PA3G, PA5F, PBA4, PBH5, and PCF5 (Faircloth 2008). After treating all DNA samples 1:1 with 10% Chelex resin (BioRad) to remove any possible inhibitor, we conducted PCR amplifications in 10 μL reaction volumes containing 1× PCR buffer, 1.5 mM $MgCl_2$, 1× BSA (Bovine Serum Albumin, New England Biolabs), 125 μM dNTPs, 0.5 μM primers (0.5 μM untagged primer; 0.05 μM CAG or M13-reverse tagged primer with 0.45 μM dye-labelled tag [HEX, FAM, NED] in the 5' end), 0.5 U of *Taq* DNA polymerase (AmpliTaq Gold®), and 40 ~ 100 ng of template DNA. PCR amplification was conducted using touchdown thermal cycling program (Don *et al.* 1991) encompassing 10°C span of annealing temperatures (ranges of 60-50°C or 65-55°C), with the following program: one cycle of 5 min at 95°C followed by 20 cycles of denaturation at 95°C for 20s, primer annealing at 60 or 65°C for 30s minus 0.5°C per annealing cycle, and elongation at 72°C for 90s followed by 20 cycles at 95°C for 20s, 50 or 55°C for 30s, and 72°C for 90s. A final elongation step at 72°C for 10 min was added followed by cooling to 4°C. Microsatellite PCR products were analyzed using an automated DNA sequencer (ABI 3730) with ROX500 fluorescent size standard and fragments were scored using GENEMAPPER v.4.0 (Applied Biosystems). To assess the rate of microsatellite genotyping

errors (Bonin *et al.* 2004; Hoffman & Amos 2005), 50 individual samples (representing approximately 10% of the total) were randomly chosen and regenotyped with all 16 loci. The overall error rate across all loci was 0.012. Using GMCONVERT v.0.32 (Faircloth 2006) and CONVERT v.1.31 (Glaubitz 2004), we reformatted microsatellite data from output files produced by GENEMAPPER v.4.0 (Applied Biosystems) into formats commonly used in downstream analyses.

Genetic Diversity and Bayesian Phylogenetic and Network Analyses of mtDNA

Basic diversity parameters such as haplotype diversity (h) and nucleotide diversity (π) were calculated for each local population or subspecies using ARLEQUIN v.3.11 (Excoffier *et al.* 2005). Bayesian phylogenetic inference was performed using MRBAYES v.3.1.2 (Huelsenbeck & Ronquist 2001) to infer haplotype genealogies. The Tamura-Nei nucleotide substitution model with gamma-distributed rate variation across sites and estimated proportion of invariable sites (TrN+I+Γ) was selected as the optimal model using the Akaike Information Criterion (AIC) in MODELTEST v.3.7 (Posada & Crandall 1998). Two independent runs were performed, each with four Markov Chain Monte Carlo (MCMC) samplings from 10^6 generations, and trees were sampled every 100 generations. Convergence onto the stationary distribution was verified by checking if the average standard deviation of split frequencies was below 0.05 between two independent runs. Bayesian posterior probabilities were estimated by constructing the majority-rule consensus tree among the last 750 000 generations after discarding the first 250 000 generations as burn-in. A median-joining network analysis was also performed to explore the relationships of the northern bobwhite mtDNA control region haplotypes, using NETWORK v.4.5 (http://www.fluxus-technology.com). Networks can better depict relationships among the

sampled haplotypes at the intraspecific level than phylogenetic methods because networks allow for concurrent existence of extant ancestral and descendant haplotypes (Bandelt *et al.* 1999; Posada & Crandall 2001).

Microsatellite Analysis of Genetic Diversity

Genetic variation was estimated over all 16 microsatellite loci within each population in terms of observed and expected heterozygosities (H_O and H_E), and number of alleles per locus (A) using GENETIX v.4.05 (Belkhir *et al.* 2001). The observed number of alleles in a sample may be strongly dependent on sample size, so to compare the allelic richness in our different populations, we computed allelic richness (A_C) based on a minimum size of seven individuals per population, using the rarefaction procedure implemented in FSTAT v.2.9.3 (Goudet 1995). Departures from Hardy-Weinberg equilibrium (HWE) for each locus and each population were tested for both heterozygote deficiency and heterozygote excess using a Markov chain method implemented in web-based GENEPOP v.3.4 (Raymond & Rousset 1995b). Linkage disequilibrium (LD) for each pair of loci in each population was examined using the exact probability test in GENEPOP v.3.4. Controlling for multiple tests, we used the sequential Bonferroni procedure at the significant level of 0.05 unless stated otherwise (Rice 1989).

Population Genetic Structure and Patterns of Diversifications

Levels of differentiation among populations based on mtDNA haplotypes were assessed using pairwise F_{ST} and hierarchical analyses of molecular variance (AMOVA, Excoffier *et al.* 1992). A distance matrix with the Tamura and Nei (1993) model was used for pairwise F_{ST} and AMOVA because this model is appropriate to describe the evolution of mtDNA control region

sequences. We based a priori groupings for AMOVA on several scenarios to distinguish whether differences among populations and subspecies are better explained by current subspecies limits or geographical proximity. Each procedure for pairwise F_{ST} and AMOVA was repeated for 10,000 random permutations to assess significance. Additionally, SAMOVA v.1.0 (spatial analysis of molecular variance) was used to define groups of genetically homogeneous or heterogeneous populations (Dupanloup *et al.* 2002). We identified the most likely number of groups (K) by running the SAMOVAs for $K = 2$ to 10, by comparing the proportions of explained variance due to among-groups, and by retaining the largest value among them (Dupanloup *et al.* 2002). For the microsatellite data, we used GENEPOP v.3.4 and SPAGEDI v.1.2 (Hardy & Vekemans 2002) to detect global and pairwise differentiation on microsatellite allele frequencies among populations (Raymond & Rousset 1995a). The allele size permutation test was then used to assess if allele sizes are important to population differentiation (Hardy *et al.* 2003), with 10 000 permutations of allele sizes implemented in SPAGEDI v.1.2. The rejection of the null hypothesis of $R_{ST} = pR_{ST}$ (estimate of F_{ST}) would suggest that the mutation process follows a stepwise mutation model (SMM) and that R_{ST} measures are considered to better reflect the genetic differentiation in this study (Slatkin 1995; Balloux & Goudet 2002; Balloux & Lugon-Moulin 2002). When the null hypothesis is not rejected, F_{ST} measure is considered to better estimate of the genetic differentiation because this measure has reduced variance, particularly, in weakly structured populations (Balloux & Goudet 2002). To investigate patterns of genetic structure among sampled populations in relation to geographic distribution, we conducted the Mantel test, using the web-based IBDWS v.3.15 (Jensen *et al.* 2005). For this analysis, we regressed pairwise estimates of $F_{ST}/(1 - F_{ST})$ or $R_{ST}/(1 - R_{ST})$ based on both mtDNA sequences and microsatellite alleles against the geographic distance (Rousset 1997).

For the microsatellite data, we also used a Bayesian model-based clustering procedure to infer population structure and to assign individuals to populations. Based on allele frequencies, this method identifies the number of *K* unknown genetic populations in which the sampled multilocus genotypes can be split and simultaneously individuals are probabilistically assigned to the original population or to more than one population if they are admixed. We used STRUCTURE v.2.2.3 to detect genetically distinct populations and assign the individuals to the populations, using admixture model, which assumes mixed ancestry of individuals, and correlated allele frequencies, which assumes that allele frequencies in the different populations are likely to be dependent due to migration or shared ancestry (Pritchard *et al.* 2000; Falush *et al.* 2003). We performed five independent runs, specifying that the numbers of populations (*K*) from 1 to 10. For each run, we ran the MCMC of 10^6 steps after a burn-in of 100 000. The value of *K* best fitting our dataset was selected both using log posterior probability of the data for a given *K*, Ln*Pr(X|K)*, and the rate of change in the log posterior probability of data between successive *K*, Δ*K*, as described in Evanno *et al.* (2005). Graphical representation of membership proportions was generated by DISTRUCT v.1.1 (Rosenberg 2004). Patterns of differentiation were also generated and visualized by a factorial correspondence analysis (FCA) of individual multilocus genotypic scores using GENETIX v.4.05.

RESULTS

Genetic Diversity and Bayesian Phylogenetic and Network Analyses of mtDNA

The mtDNA control region sequence alignment (600 bps) from 273 northern bobwhites showed 60 different haplotypes, defined by 32 polymorphic sites. Among populations with ≥7 individuals analyzed, both haplotype and nucleotide diversity were high in continuously

widespread populations, ranging from 0.622 in New Jersey to 0.946 in Kentucky-Tennessee for haplotype diversity and from 0.12% in New Jersey to 0.48% in New York and Kansas-Oklahoma for nucleotide diversity; whereas diversity in the isolated Arizona population was low, showing 0.503 for haplotype and 0.08% for nucleotide diversity (Table 4.1). Bayesian phylogenetic (Fig. 4.2) and median-joining network (Fig. 4.3) analyses of the mtDNA control region resolved two geographically divided phylogroups (widespread eastern populations and isolated Arizona population). However, there was no clear pattern of substructure among widespread eastern populations. The most common haplotype (H13) was found in 66 individuals (24.2% of all samples, Figure 4.2). This haplotype was dominant in populations representing four putative subspecies (*C. v. marilandicus*, *C. v. virginianus*, *C. v. mexicanus,* and *C. v. taylori*), but was not identified in *C. v. ridgwayi*, *C. v. texanus* and *C. v. floridanus* (Figure 4.2). Two haplotypes (H1 and H2) were found in only the isolated Arizona population and there was no other haplotype observed in Arizona. A clade of haplotypes (H9, H10 and H12) was observed in only subspecies *C. v. floridanus* (Figure 4.2), and all but one among them were only in southern Florida (FLs). Excluding these haplotypes, all others (with at least five of frequency) were widespread haplotypes across the range.

Microsatellite Diversity and Hardy-Weinberg and Linkage Equilibrium

All loci were polymorphic and revealed 243 alleles from 16 loci using 513 northern bobwhite samples. Allele number ranged from 3 for loci P2D7 and PBH5 to 29 alleles observed for locus PA12A (average 15.2 alleles per locus). Average number of alleles per locus among populations ranged from 4.75 in Arizona to 10.19 in Florida-Georgia and allelic richness ranged from 3.60 in Arizona to 5.65 in Mississippi-Tennessee based on a minimum size of seven individuals per

population (Table 4.1). This translated into average H_E ranging from 0.54 in Arizona to 0.71 in Florida-Georgia and Mississippi-Tennessee. The lowest degree of heterozygosity, mean number of alleles and allelic richness were detected in isolated Arizona population (Table 4.1).

Significant departures from Hardy-Weinberg equilibrium (HWE) were inferred for 14 of 19 populations across all loci (Table 4.1). HWE in population-by-microsatellite locus, however, was not rejected in all but 35 of 304 tests (11.5%) after sequential Bonferroni correction. Departure from HWE may be due to null alleles because 31 of these 35 violations showed tendencies for heterozygote deficiency (*U*-test, Rousset & Raymond 1995). These cases of disequilibrium were not concentrated at a single locus, or a single population. Average F_{IS} was 0.09 across all loci. Linkage disequilibrium was significant for 58 of 2280 locus pairs for all populations after sequential Bonferroni correction. Among these deviations, 48 cases were found in the New Jersey population (40% of New Jersey locus pairs). After removing the New Jersey population, all locus combinations were in linkage equilibrium in each population but 10 locus combinations (0.4% of all remaining locus combinations). Significant values involved different pairs of loci and occurred in 7 different populations. We removed the New Jersey population from all microsatellite analyses unless stated otherwise.

Population Genetic Structure and Patterns of Diversifications

Tests for genetic differentiation on mtDNA haplotypes indicated significant population structure across populations (overall pairwise $F_{ST} = 0.346$, $P < 0.001$). The isolated Arizona population was significantly differentiated from all other populations even after sequential Bonferroni correction (Table 4.2). The southern population in Florida (FLs) was distinct from all others except for two populations (New York and eastern Kansas), but the central Florida population

(FLc) was not genetically isolated from others except for five populations (North Carolina, Iindiana, Missouri, southern Florida, and Arizona). It was noted that central and southern Florida populations were genetically separated each other (Table 4.2). However, all other populations across eastern range of northern bobwhites were genetically homogeneous with low F_{ST} values after sequential Bonferroni correction. A pattern of genetic structure across eastern range of populations in relation to geographic distribution demonstrated a weak but significant correlation, explaining about 6% of the variance (one-tailed $P < 0.05$; 30,000 randomizations; Figure 4.4a). When populations were assigned to seven groups, corresponding to seven subspecies, AMOVA showed that 36% of the total genetic variance was explained by the variation among subspecies (Table 4.3). AMOVA under the model of two geographical regions (widespread eastern populations and the isolated Arizona population) indicated that up to 74% of the total genetic variance was explained by the variation among two geographical groups (Table 4.3). However, the proportion of explained variance by the hierarchical AMOVA under the subspecies model excluding *C. v. ridgwayi* was less than 10%, suggesting the potential of high gene flow and limited genetic structure among widespread eastern populations. Based on populations with at least seven individuals, SAMOVA confirmed that there were two genetically distinct groups ($K = 2$, a widespread eastern group and an isolated Arizona population) in northern bobwhites in USA (Table 4.4). The proportion of explained variance by SAMOVA decreased as the number of groups increased, ranging from 74% ($K = 2$) to 44% ($K = 10$). However, it was revealed that southern and central populations in Florida were not grouped into a single cluster in all SAMOVAs of $K = 2$ to 10. The most genetically isolated populations were the Arizona population, and then followed by those in southern Florida and New York in sequential SAMOVA algorithms (Table 4.4).

Global differentiation across the range was confirmed with microsatellite allele frequencies (F_{ST} = 0.043 and R_{ST} = 0.068, $P < 0.05$). The global R_{ST} value was significantly higher than pR_{ST} ($P < 0.05$), suggesting that the R_{ST} measure was expected to better reflect genetic characteristics than F_{ST} for this study. Most pairwise R_{ST} among local populations were not significant even though several R_{ST} values for some comparisons were relatively high (e.g., Arizona vs New York, Table 4.2). However, pairwise genetic distance among populations, $R_{ST}/(1-R_{ST})$, was positively correlated with their corresponding geographic distance, explaining 32% of variance (one-tailed $P < 0.0001$; 30,000 randomizations; Figure 4.4b).

Bayesian clustering procedure indicated that $K = 5$ was the most probable number of groups found across the study area with the highest values of both $\mathrm{Ln}Pr(X|K) = -22649.2$ and $\Delta K = 54.05$ (Figure 4.5). For $K > 5$ or $K < 5$, $\mathrm{Ln}Pr(X|K)$ values decreased and variance among five independent runs was larger (Figure 4.5). However, the proportion of membership of each sampled population in each of the five groups did not have a simple geographical interpretation for this model of $K = 5$. All populations in widespread eastern range comprised individuals with higher levels of admixture, whereas the isolated Arizona population was assigned 90.6% of their individuals into a single cluster under the model of $K = 5$ (Figure 4.6). In contrast, more clear genetic differentiation between the isolated Arizona population and widespread populations across eastern range was visualized for $K = 2$ (Figure 4.6), suggesting that this was the major differentiation in the whole study area. This clear genetic structure in the range was also supported by a factorial correspondence analysis (FCA) of individual multilocus genotypic scores (Figure 4.7). The first and the second axis of the FCA explained 21.2% and 9.8% of the total variation at the population level, and 41.5% and 21.3% at the subspecies level. Individuals

from the isolated Arizona population were clearly clustered, but those from the eastern widespread range appeared to form a single cluster, rather than multiple groups.

DISCUSSION

Our major finding was, in both analyses of mitochondrial and nuclear microsatellite markers, extremely high genetic differentiation between isolated Arizona northern bobwhites (masked bobwhite, *C. v. ridgwayi*) and the others. In contrast, the other populations across a large and continuous geographical range in Midwestern and eastern USA were not substantially different from each other, showing little genetic differentiation, with some possible exceptions.

Bayesian phylogenetic and network analyses of mtDNA sequences grouped all Arizona haplotypes into a monophyletic clade and clearly identified the other main mtDNA haplogroup, which has widespread distributions in Midwestern and eastern USA. Pariwise F_{ST} values among populations and AMOVA results also confirmed this deep genetic structure. The analyses that corroborated the main distinction between the isolated Arizona population and the others were a Bayesian model-based clustering analysis (STRUCTURE) in the basis of microsatellite loci. This method has the advantage of using individuals as the unit of analysis, allowing the inference of population structure without information on predefined sampling location of individuals (Pritchard *et al.* 2000; Falush *et al.* 2003). In our analysis, although both posterior probability and the statistic, ΔK, of the Bayesian clustering analysis indicated a congruent signal for the existence of five differentiated groups ($K = 5$), only one group was able to be associated to a particular geographical population, Arizona, with correctly assigning more than 90% of Arizona individuals to this group. In contrast, northern bobwhites across the widespread range including Midwestern and eastern USA were not assigned to any specific or geographical group but were

dispersed in the other four groups. It is noted that the posterior probability procedure implemented in STRUCTURE tends to overestimate the number of clusters (Waples & Gaggiotti 2006) and that using ΔK may be more appropriate to estimate the true number of clusters particularly when patterns of gene flow among populations are not homogeneous (Evanno *et al.* 2005). Considering this, our microsatellite data suggests a mixed model for northern bobwhite genetic structure in USA, consisting of an isolated distinct group in Arizona and a hierarchical set of a few overlapping groups across the widespread range of eastern populations. A factorial correspondence analysis also supported this finding identifying masked bobwhites in Arizona (*C. v. ridgwayi*) as a single cloud and the others across Midwest and eastern USA as a distinct cluster.

North America is divided into two main areas by continuous mountain ranges. This continental dividing ridge line runs from northwestern Canada along the peaks of the Rocky Mountains in USA, then into Mexico along the crests of the Sierra Madre Occidental. Restriction effect of these high ridges on northern bobwhite dispersal and thereby gene flow may be associated with this deep genetic differentiation between isolated Arizona and the other continuous populations. This pattern has been documented in other widespread North American birds, and many co-distributed taxa on one side divided by this barrier have shown morphological, ecological, behavioral, and other life-history distinctions from their relatives on the other side (see references in Avise 2000). Habitat features may also play a critical role in genetic divergence at this continental scale. The northern bobwhite is generally grassland-preferred and forest edge-adapted species, but masked bobwhite in Arizona is relatively more adapted to hot, deep grasslands in more xeric or desert environment characterized pronounced precipitation peaks that occur during late summer (Johnsgard 1988; Carroll 1994; Brennan 1999; Hernandez *et al.* 2006). These habitat differences may result in natal imprinting for particular

environment and prevent birds in both areas from migrating across habitats and interbreeding each other (Davis & Stamps 2004; Hull *et al.* 2008). Differences in habitat environments may result in life history modifications between masked bobwhites and other subspecies. For example, masked bobwhites initiate breeding much later (June for masked bobwhite vs March for other subspecies) and experience a much shorter (90 days for masked bobwhite vs 150 days for other subspecies) breeding season compared with Midwestern and eastern subspecies, and this shortened nesting season can limit masked bobwhites' reproduction potential (Brennan 1999; Hernandez *et al.* 2006). Given this severe hot and dry environment, low dispersal rate, and habitat loss or fragmentation of the masked bobwhite, it is not surprising that masked bobwhite of Arizona has the lowest values in all genetic diversity information of both mitochondrial and microsatellite loci (Table 4.1). It is not difficult to comprehend that this low genetic diversity is certainly associated with low fitness, reduction in population size, and therefore why masked bobwhite was listed as endangered by the U.S. government and was faced by local extinction.

Our results suggest that populations in Midwestern and eastern USA are genetically homogeneous, with the possible exception of *C. v. floridanus*. Relatively high and significant pairwise mtDNA F_{ST} values between the southern Florida population (FLs) and the other populations, coupled with SAMOVA results, imply that this population of *C. v. floridanus* is genetically separable from other populations or subspecies, even from a central Florida population (FLc) in the same subspecies. This suggests that genetic differentiation among populations within *C. v. floridanus* may be greater than genetic divergence across different subspecies. However, except for masked bobwhites, mtDNA haplotypes did not show reciprocally monophyletic structure for any single subspecies or populations including FLs. Although there was a signal for multiple distinct genetic groups in Midwestern and eastern birds

based on the Bayesian clustering method using microsatellite loci, this possible substructuring was not directly associated with geographical or taxonomic delineation. Pairwise R_{ST} values from Microsatellite loci also indicated that the levels of genetic divergence among populations in Midwestern and eastern USA were relatively quite low compared to the levels of Arizona and the other populations. Although the species was known as one of the least mobile residents in Galliformes (Stoddard 1931), radiotelemetry analyses have shown that northern bobwhites are capable of moving among habitats separated by 1-3 km (Fies *et al.* 2002; Townsend *et al.* 2003). In the Smoky Mountains across Tennessee and North Carolina, elevational movements between breeding and wintering sites are also known (Rosene 1969; Brennan 1999). Such a level of dispersal can result in genetic homogenization among populations (Hernandez *et al.* 2006).

Our analyses of both mitochondrial and microsatellite data revealed little genetic structure in eastern widespread range, largely due to different frequencies of widespread haplotypes or alleles rather than due to lineage specific private haplotypes or alleles. However, there was a significant positive relationship between genetic and geographic distance, suggesting that populations were in equilibrium between gene flow and genetic drift (Hutchison & Templeton 1999). Recent widespread colonization processes may reflect little genetic substructure of northern bobwhites in the Midwestern and eastern range, with some common haplotypes or alleles having evolved before the current population structure had been formed (Bulgin *et al.* 2003). In the 18th and 19th centuries, the northern bobwhite rapidly extended its range into the Midwest where formerly the vast expanses of grass severely limited its usefulness for the species, with deforestation and cultivation of northeastern U.S. by European settlers (Forbush 1912; Edminster 1954; Bent 1963). This colonization process and current widespread distribution, coupled with the apparent pattern of isolation by distance, suggest they have not

diverged enough to reach complete lineage sorting or genetic distinction among populations in this range.

Only two individuals of *C. v. texanus* were analyzed with mtDNA control region and they had different haplotypes. One of them was distinct from two main haplotype groups, but the other was interspersed in the haplotype group for the widespread range. To better understand genetic structure and diversity of the *C. v. texanus*, and its relationships with other subspecies, it should be analyzed more thoroughly with additional samples.

Although our results based on mitochondrial and nuclear microsatellite markers indicated congruent patterns discussed above, our study revealed contrasting patterns of mitochondrial and nuclear microsatellite markers on estimated extent of overall and pairwise genetic differentiation among populations. Such conflicting results between these independent data have been also found in many other studies (e.g., Haavie *et al.* 2000; Johnson *et al.* 2003; Brito 2007; Kawakami *et al.* 2007). The difference is explained by the hypothesis that the effective population size of maternally (e.g., mtDNA) and biparentally (e.g., nuclear microsatellites) inherited markers can greatly influence the level of genetic structure. It is generally assumed that the effective population size of uniparentally inherited marker is four times less than in biparentally inherited markers (Seielstad *et al.* 1998). Despite the values from both markers are not directly comparable to each other due to inheritance mode or mutation rate, we compared transformed F_{ST} estimates (following by the equation, $F_{ST(nuclear)} = F_{ST(mitochondrial)}/(4 - 3F_{ST(mitochondrial)})$, assuming an infinite island model at mutation-drift equilibrium with no sex-biased dispersal and sex-ratio of one (Wright 1951; Birky *et al.* 1983; Crochet 2000; Brito 2007). Comparing overall F_{ST} estimates from microsatellites and mtDNA data for northern bobwhites still revealed greater mitochondrial than nuclear genetic structure ($F_{ST} = 0.043$ from

microsatellites and corrected F_{ST} = 0.128 from mtDNA). This suggests that there could be other effects beyond low effective population size and genetic drift effect for the mitochondrial marker, when we explain differences in northern bobwhite genetic structure between two marker systems.

Another explanation is the relative importance of mutation process and rate for microsatellite markers. For this, we analyzed genetic differentiation using R_{ST} under the stepwise mutation model (Ohta & Kimura 1973; Kimura & Ohta 1978) and tested for the importance of allele size of microsatellite loci (Hardy *et al.* 2003). We found that a significantly greater value of R_{ST} than pR_{ST} (computed R_{ST} after allele-size permutation), indicating that the stepwise mutation model reflecting information on allele size was informative for this genetic structuring (Hardy *et al.* 2003). In addition to the mutation process, mutation rate of marker systems could be significant. Generally, low mutation rate may not be enough to detect existing genetic divergence; however, fast mutation rate of microsatellite loci can even cover the signal for high differentiation among populations (Hedrick 1999; Brito 2007). Thus, our contrasting pattern in mitochondrial and microsatellite results can be explained by both the stepwise mutation process and the fast rate of microsatellite loci.

Finally, we suggest that male-biased gene flow can explain the increased genetic divergence observed in mtDNA relative to microsatellite loci. If males are more mobile and females are more philopatric, maternally inherited mitochondrial DNA can indicate increased genetic structure compared to biparentally inherited microsatellites (Gibbs *et al.* 2000; Prugnolle & de Meeus 2002). Our results also revealed this pattern, as frequently shown in other animals (e.g., Melnick & Hoelzer 1992; Gibbs *et al.* 2000). This hypothesis is supported by previous radiotelemetry studies analyzing dispersal of northern bobwhites. For example, Fies *et al.* (2002) showed that male juveniles were more likely to disperse than female juveniles in Virginia in

winter and breeding seasons. Avian gene flow is usually the result of juveniles dispersing from their natal areas and, less frequently, adults changing their breeding locations (Johnson & Gaines 1990). Also, Townsent *et al.* (2003) indicated that males were more likely than females to move >2 km in Oklahoma during the breeding season. Therefore, our genetic results coupled with some field observation data suggest that male-biased dispersal and male-driving gene flow could explain the differences in the magnitude of differentiation between mitochondrial and microsatellite genetic structure.

IMPLICATIONS FOR TAXONOMY AND CONSERVATION

Our study is the first to use both mitochondrial and nuclear microsatellilte loci to examine patterns and levels of genetic differentiation across the widespread northern bobwhite. Observed patterns of genetic diversity and structure showed that the geographically isolated Arizona population (masked bobwhite, *C. v. ridgwayi*) was at low genetic diversity and extremely differentiated from the other subspecies and populations. In contrast, the other widespread populations in midwestern and eastern USA were at relatively high genetic diversity and genetically homogeneous. The results reflect that the masked bobwhite has experienced a severe range contraction and decline in population size and therefore why the subspecies has been faced with local extinction. Based on our genetic information, in conjunction with distinct morphology and isolated geographic ranges, our analyses suggest that the masked bobwhite should be considered as a distinct subspecies and a distinct unit for conservation and management. In contrast, the other populations across six subspecies were not substantially different from each other, with some possible exceptions of Florida populations (*C. v. floridanus*). Despite the levels of genetic divergence were not strong in comparison to those of masked bobwhites, populations

across the ranges of *C. v. floridanus* need to be considered as a separate subspecies or a distinct unit for conservation and management. For *C. v. virginianus*, *C. v. marilandicus*, *C. v. mexicanus,* and *C. v. taylori*, we recommend that they be considered a single management unit because levels of genetic divergence among these putative subspecies were quite low with higher levels of admixture. However, to clarify these implications for taxonomy and conservation, it is highly recommended that further ecological studies as well as genetic research should be undertaken.

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Table 4.1: Northern Bobwhite (*Colinus virginianus*) collection sites (mapped in Figure 4.1) and descriptive genetic diversity data of each local populations based on mtDNA control region sequences and 16 multilocus microsatellite loci. Total number of individuals analyzed (N_T), number of mtDNA-sequenced individuals (n_{mt}), number of mtDNA haplotypes (n_h), haplotype (h) and nucleotide (π) diversities (± standard deviation, *SD*), number of genotyped individuals (n_{mic}), mean number of alleles per locus (A), allelic richness (A_C), observed (H_O) and expected (H_E) heterozygosities (± *SD*).

		mtDNA				Microsatellite data				
	N_T	n_{mt}	n_h	$h \pm SD$	$\pi \pm SD$	n_{mic}	A	A_C	$H_O \pm$ SD	$H_E \pm$ SD
C. v. marilandicus										
New York (NY)	24	7	4	0.857 ± 0.102	0.0048 ± 0.0033	24	5.88	4.39	0.625 ± 0.266	0.628 ± 0.234
New Jersey (NJ)	25	10	3	0.622 ± 0.138	0.0012 ± 0.0011	25	7.38	5.24	0.620 ± 0.266	0.681 ± 0.228
Eastern Virginia (VAe)	32	16	7	0.850 ± 0.060	0.0044 ± 0.0028	32	8.00	5.37	0.612 ± 0.234	0.689 ± 0.244
C. v. virginianus										
Southern Virginia (VAs)	30	.	.	.	.	30	8.63	5.58	0.664 ± 0.257	0.703 ± 0.230
North Carolina (NC)	28	28	12	0.897 ± 0.031	0.0038 ± 0.0024	26	7.63	5.33	0.708 ± 0.234	0.694 ± 0.208
South Carolina (SC)	4	4	4	1.000 ± 0.177	0.0050 ± 0.0039	.	.	.	.	.
Florida-Georgia (FLGA)	60	11	6	0.727 ± 0.144	0.0022 ± 0.0017	60	10.19	5.61	0.626 ± 0.232	0.712 ± 0.220
C. v. floridanus										
Central Florida (FLc)	28	16	6	0.733 ± 0.102	0.0026 ± 0.0018	28	7.56	5.15	0.584 ± 0.271	0.664 ± 0.245
Southern Florida (FLs)	24	9	4	0.694 ± 0.147	0.0024 ± 0.0018	24	6.50	4.92	0.609 ± 0.295	0.676 ± 0.220
C. v. mexicanus										
Illinois (IL)	10	10	4	0.644 ± 0.152	0.0016 ± 0.0013	.	.	.	.	.
Indiana (IN)	26	19	10	0.912 ± 0.040	0.0038 ± 0.0024	26	7.63	5.26	0.634 ± 0.236	0.683 ± 0.237
Central Kentucky (KYc)	23	6	6	1.000 ±	0.0041 ±	23	7.69	5.32	0.619 ± 0.291	0.662 ± 0.253

				0.096	0.0030					
				1.000 ±	0.0034 ±					
Eastern Kentucky (KYe)	18	2	2	0.500	0.0041	18	6.00	4.82	0.710 ± 0.222	0.685 ± 0.178
Kentucky-Tennessee				0.946 ±	0.0040 ±					
(KYTN)	11	11	9	0.066	0.0027	.	.	.	.	.
Mississippi-Tennessee				0.923 ±	0.0035 ±					
(MSTN)	25	14	10	0.060	0.0023	25	8.50	5.65	0.671 ± 0.200	0.712 ± 0.220
				0.867 ±	0.0048 ±					
Tennessee (TN)	6	6	4	0.129	0.0034	.	.	.	.	.
C. v. texanus										
				1.000 ±	0.0135 ±					
Texas (TX)	2	2	2	0.500	0.0144	.	.	.	.	.
C. v. taylori										
				0.864 ±	0.0035 ±					
Nebraska (NE)	20	12	7	0.079	0.0024	20	7.75	5.60	0.623 ± 0.269	0.695 ± 0.217
				1.000 ±	0.0017 ±					
Iowa (IA)	20	2	2	0.500	0.0024	20	7.19	5.44	0.628 ± 0.223	0.699 ± 0.229
				0.939 ±	0.0029 ±					
Central Kansas (KSc)	20	12	9	0.058	0.0020	20	7.06	5.28	0.660 ± 0.205	0.681 ± 0.224
				0.806 ±	0.0020 ±					
Eastern Kansas (KSe)	9	9	5	0.120	0.0016	.	.	.	.	.
				0.905 ±	0.0048 ±					
Kansas-Oklahoma (KSOK)	15	15	9	0.054	0.0030	12	6.31	5.44	0.672 ± 0.175	0.704 ± 0.172
				0.844 ±	0.0028 ±					
Missouri (MO)	24	10	5	0.080	0.0020	24	7.06	5.18	0.697 ± 0.247	0.701 ± 0.219
				0.851 ±	0.0046 ±					
Oklahoma-Texas (OKTX)	41	24	9	0.046	0.0028	41	8.69	5.27	0.599 ± 0.212	0.696 ± 0.199
C. v. ridgwayi										
				0.503 ±	0.0008 ±					
Arizona (AZ)	35	18	2	0.064	0.0008	35	4.75	3.60	0.476 ± 0.214	0.543 ± 0.184

Table 4.2: Pairwise values of F_{ST} (mtDNA; above the diagonal) and R_{ST} (microsatellites; below the diagonal) among populations (with seven or more individuals per population) of northern bobwhites. * indicates significant ($P < 0.05$) pairwise F_{ST} or R_{ST}, and values in bold are significant after sequential Bonderroni correction. NA, not available.

	NY	NJ	VAe	VAs	NC	FLGA	FLc	FLs	IL	IN	KYc	KYe	KYTN	MSTN	NE	IA	KSc	KSe	KSOK	MO	OKTX	AZ
NY		0.1447	0.0017	NA	0.0268	0.1567*	0.2644*	0.4969*	0.1346	0.1010*	NA	NA	0.0329	0.1052*	-0.0178	NA	0.0561	0.0341	0.0004	0.121	0.015	**0.8567***
NJ	NA		0.0715	NA	0.0059	-0.0313	0.1761*	**0.5980***	-0.0683	0.0267	NA	NA	0.022	-0.0136	0.1169	NA	-0.0512	-0.0584	-0.0067	0.1336	-0.0028	**0.9249***
VAe	0.042	NA		NA	0.0179	0.0585	0.2070*	**0.4416***	0.0877*	0.1110*	NA	NA	0.0469	0.0545	0.0558	NA	0.059	0.02	0.0254	0.1258*	0.027	**0.8013***
VAs	0.0474	NA	0.0179		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
NC	0.038	NA	-0.0005	0.035		0.0048	**0.1570***	**0.4383***	0.0204	0.0575*	NA	NA	0.0205	0.0136	0.0506	NA	0.0141	-0.0135	0.0183	0.1004*	0.0148	**0.8049***
FLGA	0.0416	NA	0.0304	0.0076	0.0464		0.1570*	**0.5335***	-0.0306	0.0198	NA	NA	0.0162	-0.0337	0.1274*	NA	0.0069	-0.0029	0.015	0.1557*	0.0074	**0.8970***
FLc	0.0558	NA	0.0087	0.0294	0.0205	0.0376		**0.3398***	0.1766*	**0.1925***	NA	NA	0.1714*	0.1246*	0.2243*	NA	0.1612*	0.1786*	0.1303*	**0.2639***	0.1221*	**0.8644***
FLs	0.0959	NA	0.0603	0.0624	0.0554	0.0647	-0.0016		**0.5732***	**0.4677***	NA	NA	**0.4439***	**0.4227***	**0.4754***	NA	**0.4804***	0.5503*	**0.4004***	**0.5511***	**0.3890***	**0.8909***
IL	NA	NA	NA	NA	NA	NA	NA	NA		-0.006	NA	NA	-0.0019	-0.0435	0.0909	NA	-0.0235	-0.0086	0.0044	0.1214	-0.0094	**0.9146***
IN	0.0464	NA	-0.0088	0.0105	0.0115	0.0245	-0.0003	0.0351	NA		NA	NA	-0.003	0.0121	0.0775*	NA	0.0266	0.0187	0.0376	0.1080*	0.0315	**0.8359***
KYc	0.0346	NA	0.0188	0.0216	0.0487	0.0283	-0.0054	0.0105	NA	-0.0066		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
KYe	0.0775	NA	0.0033	0.0177	0.0127	0.0264	-0.0012	0.0438	NA	0.0183	0.0319		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
KYTN	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		-0.0027	-0.0009	NA	0.0001	-0.0084	0.009	0.0862	-0.0029	**0.8496***
MSTN	0.0206	NA	-0.0059	0.0264	0.0062	0.0278	0.0043	0.0485	NA	0.0047	0.0247	0.0184	NA		0.0584	NA	0.01	0.0057	0.0083	0.0818	-0.0077	**0.8460***
NE	0.0738	NA	0.0504*	0.0342	0.0637	0.0369	0.011	0.0092	NA	0.0256	0.0103	0.0374	NA	0.0339		NA	0.055	0.0404	0.0312	0.1179	-0.0005	**0.8442***
IA	0.069	NA	0.0312	0.0207	0.0502	0.0261	0.0055	0.0028	NA	0.0047	-0.005	0.024	NA	0.029	-0.0243		NA	NA	NA	NA	NA	NA
KSc	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		-0.0797	-0.0307	0.08	-0.0022	**0.8782***
KSe	0.0612	NA	0.0069	0.0095	0.0232	0.0168	0.0176	0.0636	NA	0.0053	0.0317	0.0398	NA	-0.0052	0.006	0.0098	NA		-0.0602	0.0895	-0.0312	**0.9047***
KSOK	0.1114	NA	0.0359	0.0331	0.0617	0.0461	0.0607	0.0974	NA	0.033	0.0563	0.0574	NA	0.043	0.0128	0.0116	NA	0.0034		0.0496	-0.0315	**0.8091***
MO	0.0471	NA	0.0177	0.0289	0.0139	0.0381	-0.0014	0.0094	NA	0.0091	0.019	0.0245	NA	-0.0048	-0.0076	0.004	NA	-0.0203	0.0347		0.029	**0.8740***
OKTX	0.1309	NA	0.0751	0.0602	0.0906	0.0713	0.0847*	0.1031*	NA	0.0675	0.0971*	0.0846	NA	0.0666	0.0292	0.0249	NA	0.0171	-0.0256	0.0467		**0.7751***
AZ	0.3108	NA	0.2365	0.1948	0.2894	0.1819	0.2777*	0.3137*	NA	0.2391	0.2339	0.3003	NA	0.2591	0.2326	0.1914	NA	0.2464	0.1399	0.2751	0.1702	

Table 4.3: Analysis of molecular variance (AMOVA) for the northern bobwhites. Samples were partitioned with several *a priori* grouping scenarios, based on putative subspecies and/or geographic distribution.

Grouping	Source of variation	d.f	% variation	
All seven subspecies separated	Among subspecies	6	36.32	***
	Among populations within subspecies	17	2.13	*
	Within populations	249	61.55	***
C. v. ridgwayi -	Among subspecies	1	73.72	*
the other six subspecies	Among populations within subspecies	22	2.80	***
	Within populations	249	23.48	***
C. v. ridgwayi - *C. v. texanus* -	Among subspecies	2	72.83	**
the other five subspecies	Among populations within subspecies	21	2.50	***
	Within populations	249	24.67	***
C. v. ridgwayi - *C. v. floridanus* -	Among subspecies	2	59.63	*
the other five subspecies	Among populations within subspecies	21	2.51	***
	Within populations	249	37.87	***
C. v. ridgwayi - *C. v. floridanus* -	Among subspecies	3	60.03	**
C. v. texanus -	Among populations within subspecies	20	1.78	**
the other four subspecies	Within populations	249	38.19	***
All six subspecies	Among subspecies	5	8.80	***
without *C. v. ridgwayi*	Among populations within subspecies	17	2.47	*
	Within populations	232	88.74	***

Table 4.4: Population structure inferred by spatial analysis of molecular variance (SAMOVA) based on northern bobwhite mtDNA haplotypes. Best results for each pre-defined number of groups $K = 2$ to 5 are shown.

Mode	Grouping	Source of variation	d.f	% variation	
$K = 2$	[AZ] / [the others]	Among groups	1	74.32	*
		Among populations within groups	16	2.75	***
		Within populations	233	22.93	***
$K = 3$	[AZ] / [FLs] /	Among groups	2	69.96	**
	[the others]	Among populations within groups	15	1.72	***
		Within populations	233	28.32	***
$K = 4$	[AZ] / [FLs] / [NY] /	Among groups	3	65.39	***
	[the others]	Among populations within groups	14	1.87	***
		Within populations	233	32.74	***
$K = 5$	[AZ] / [FLs] / [NY] /	Among groups	4	60.25	***
	[MO] / [the others]	Among populations within groups	13	1.94	***
		Within populations	233	37.81	***

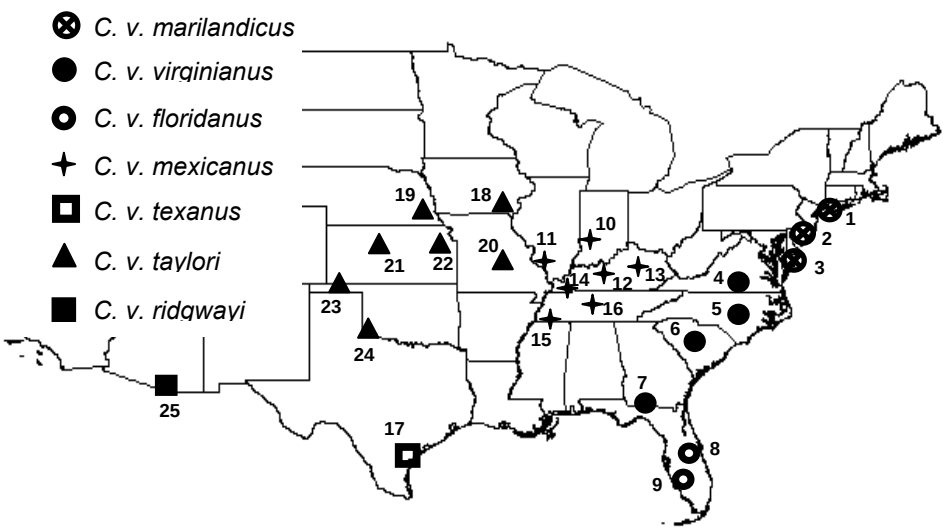
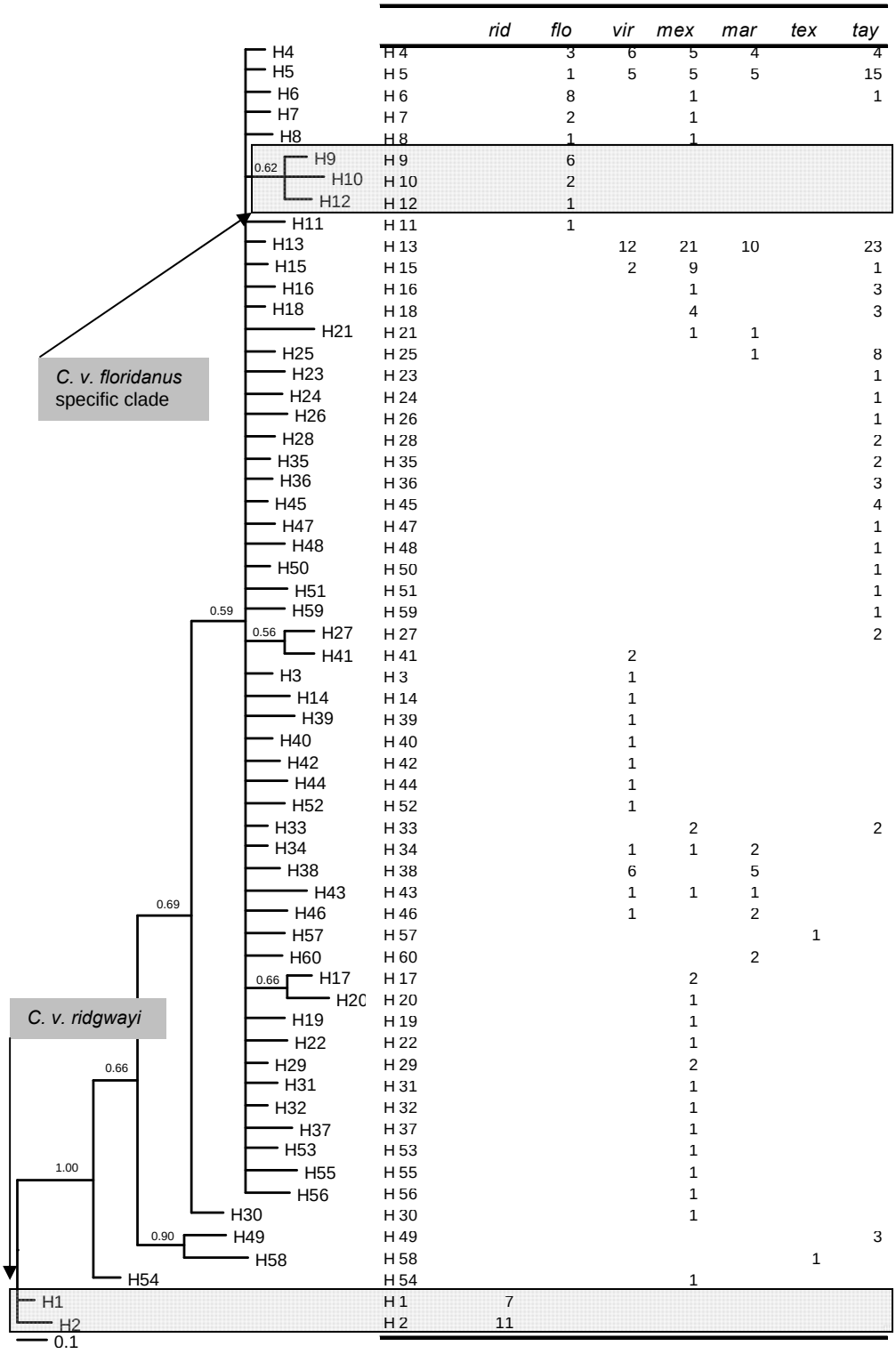


Figure 4.1: Map of sampling populations across the entire subspecies range for northern bobwhites in the USA: 1, New York (NY); 2, New Jersey (NJ); 3, Eastern Virginia (VAe); 4, Southern Virginia (VAs); 5, North Carolina (NC); 6, South Carolina (SC); 7, Florida-Georgia (FLGA); 8, Central Florida (FLc); 9, Southern Florida (FLs); 10, Indiana (IN); 11, Illinois (IL); 12, Central Kentucky (KYc); 13, Eastern Kentucky (KYe); 14, Kentucky-Tennessee (KYTN); 15, Mississippi-Tennessee (MSTN); 16, Tennessee (TN); 17, Texas (TX); 18, Iowa (IA); 19, Nebraska (NE); 20, Missouri (MO); 21, Central Kansas (KSc); 22, Eastern Kansas (KSe); 23, Kansas-Oklahoma (KSOK); 24, Oklahoma-Texas (OKTX); 25, Arizona (AZ).



	rid	flo	vir	mex	mar	tex	tay
H4		3	6	5	4		4
H5		1	5	5	5		15
H6		8		1			1
H7		2		1			
H8		1		1			
H9		6					
H10		2					
H12		1					
H11		1					
H13			12	21	10		23
H15			2	9			1
H16				1			3
H18				4			3
H21				1	1		
H25					1		8
H23							1
H24							1
H26							1
H28							2
H35							2
H36							3
H45							4
H47							1
H48							1
H50							1
H51							1
H59							1
H27							2
H41			2				
H3			1				
H14			1				
H39			1				
H40			1				
H42			1				
H44			1				
H52			1				
H33				2			2
H34			1	1	2		
H38			6		5		
H43			1	1	1		
H46			1		2		
H57						1	
H60					2		
H17				2			
H20				1			
H19				1			
H22				1			
H29				2			
H31				1			
H32				1			
H37				1			
H53				1			
H55				1			
H56				1			
H30				1			
H49							3
H58						1	
H54				1			
H1	7						
H2	11						

Figure 4.2: Bayesian inference in northern bobwhite mtDNA control region haplotypes (H1 to H60), computed using TrN+I+G genetic substitution model. Bayesian posterior probabilities are

given above the branches. The table on the right shows the designated subspecies of these haplotypes (*rid = C. v. ridgwayi; flo = C. v. floridanus; vir = C. v. virginianus; mex = C. v. mexicanus; mar = C. v. marilandicus; tex = C. v. texanus; tay = C. v. taylori*).

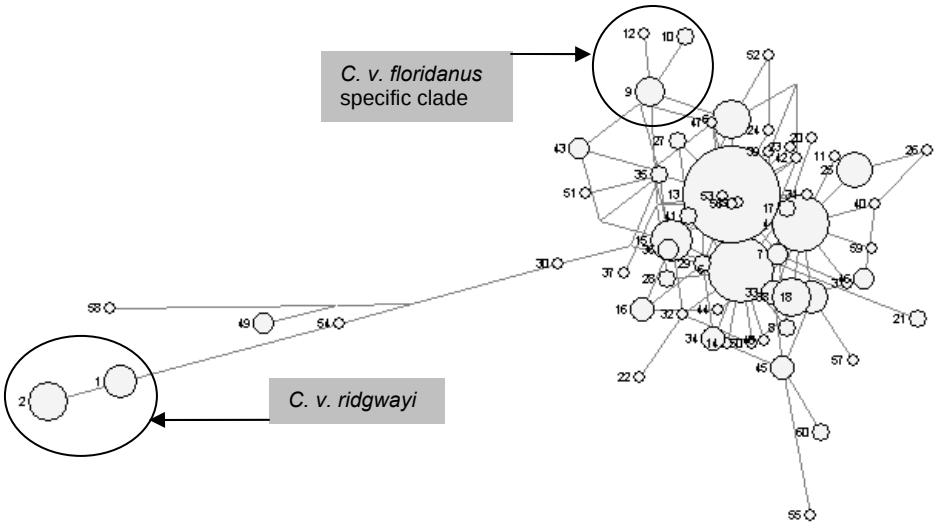


Figure 4.3: A median-joining network for 60 haplotypes of the northern bobwhites. The relative sizes of the circles represent the number of individuals contained within each haplotype which is indicated by numbers. Line lengths reflect actual branch lengths between haplotypes.

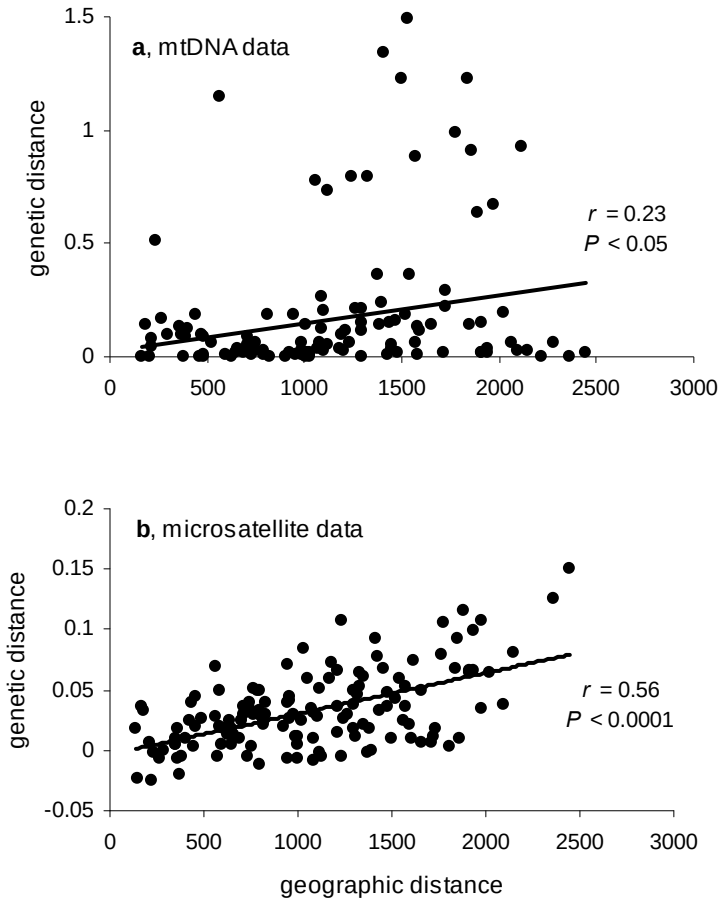


Figure 4.4: Genetic distance based on (a) mtDNA control region, $F_{ST}/(1-F_{ST})$, or (b) microsatellite data, $R_{ST}/(1-R_{ST})$, versus geographic distances, km, for pairwise combinations of northern bobwhite populations (excluding an isolated Arizona population).

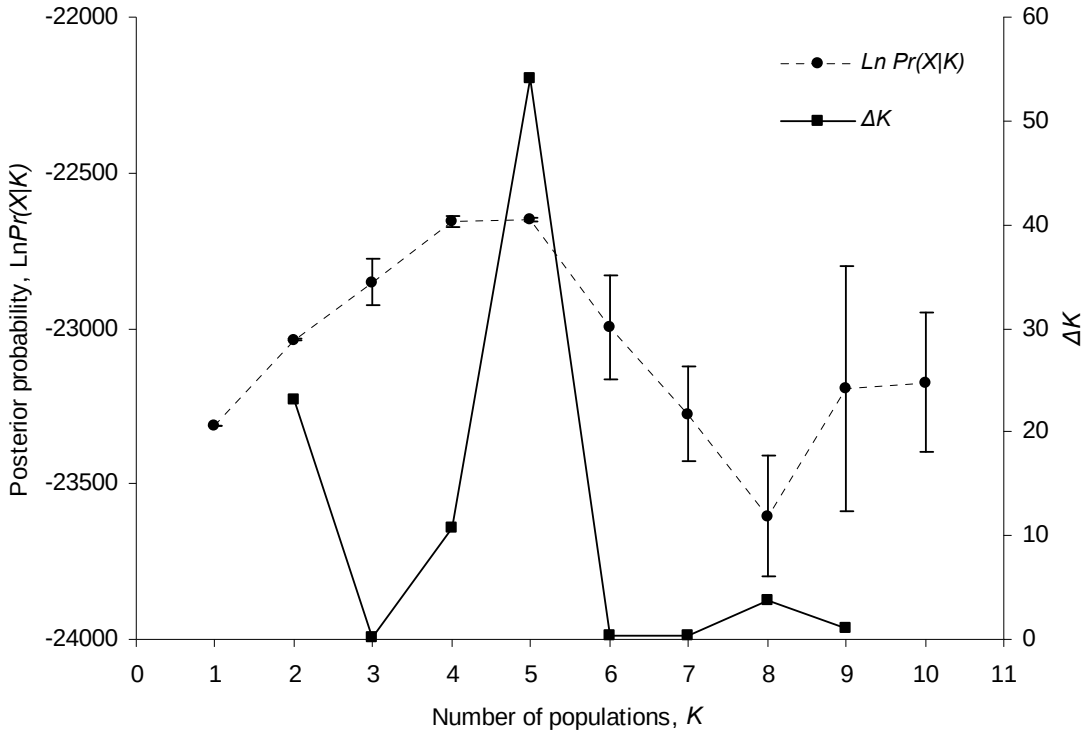


Figure 4.5: Log posterior probability of the microsatellite data for a given number of simulated cluster *K*, Ln *Pr(X|K)* with standard error (across five independent runs) and the rate of change in the posterior probability, Δ*K*, computed by STRUCTURE (under the 'admixture and correlated allele frequencies among populations' model), using the MCMC with 1 million repetitions for each run.

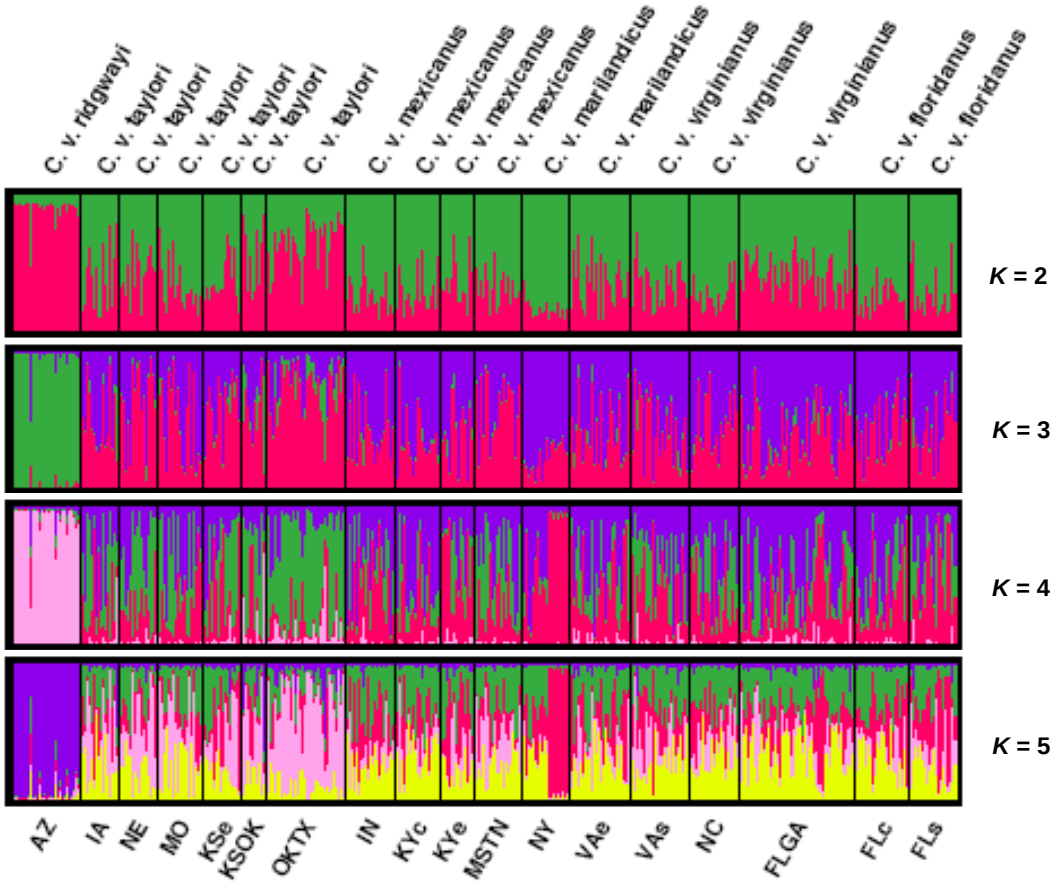


Figure 4.6: Proportion of the memberships for northern bobwhite individuals inferred with STRUCTURE v.2.2.3 and poltted with DISTRUCT v.1.1 without using prior population definitions. Each individual is represented by a vertical line partitioned into K = 2, 3, 4, and 5 clusters, respectively. Each color represents a different cluster and black lines separate the individuals of different geographically sampled populations.

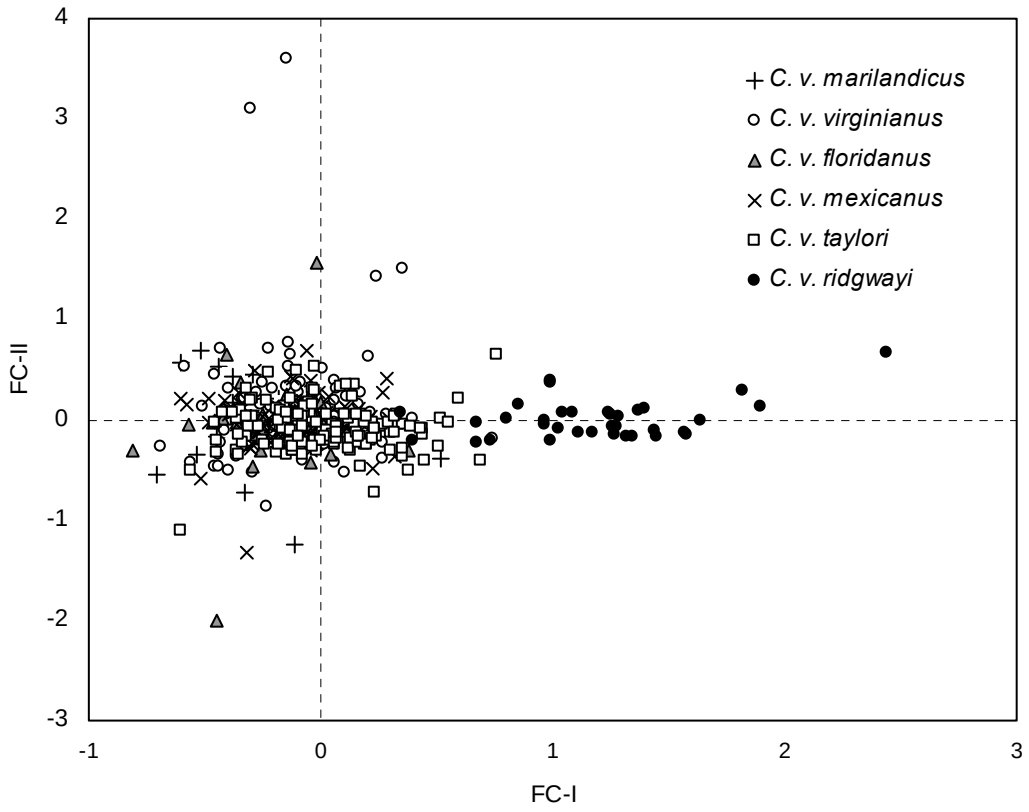


Figure 4.7: Factorial component analysis of northern bobwhite genotypes, computed by GENETIX using 16 microsatellite loci. FC-I and FC-II are the first two factorial components, explaining 41.5% and 21.3%, respectively, of the variation at the subspecies level.

CHAPTER 5

CONCLUSIONS

A large, well-resolved phylogeny, in addition to its systematic value, is an indispensable tool for testing a variety of hypotheses in ecology, evolutionary biology, and conservation biology, greatly increasing statistical power for the associated comparative analyses (Felsenstein 1985; Fisher and Owens 2004; Harvey and Pagel 1991). My Anseriformes-Galliformes phylogenetic supertree, comprising about 83% of all fowl species recognized by Dickinson (2003), represents a first attempt to derive a comprehensive species-level phylogeny of Galloanserae.

The supertree supported the partitioning of Anseriformes into the 3 traditional families Anhimidae, Anseranatidae, and Anatidae, although it showed relatively poor resolution within the Anatidae. For the Galliformes, the overall topology of majority-rule supertree was highly consistent with the hypothesis of the sequential sister-group relationships of Megapodiidae, Cracidae, and the remaining Galliformes. Overall, the supertree was well resolved, but the degree of resolution varied across the families. Areas where the supertree was either poorly resolved or incomplete reflected gaps in the existing phylogenetic information and highlighted areas in need of more study.

My species-level supertree showed that more than 30% of analyzed polytypic genera were not monophyletic. This suggests that existing results from genus-level comparative studies using the average of species should be interpreted with caution until analogous species-level comparative studies are available. Like any phylogenetic hypothesis, this supertree is naturally

open to further revision and resolution. In the meantime, however, it will provide a valuable foundation to understand the diverse biology of Galloanserae in a robust phylogenetic framework.

The role of genetics in conservation biology is diverse but, below the species-level, assessing genetic variability, resolving patterns of population structure, and identifying distinct genetic units are essential for the conservation and management of natural populations (Avise 2000; Crandall et al. 2000; Frankham et al. 2002; Moritz 1994). In analyses of both mitochondrial and nuclear microsatellite loci for northern bobwhite *Colinus virginianus*, we found extremely high genetic differentiation between isolated Arizona northern bobwhites (masked bobwhite, *C. v. ridgwayi*) and the other subspecies (*C. v. marilandicus*, *C. v. virginianus*, *C. v. floridanus*, *C. v. mexicanus*, *C. v. taylori*, and *C. v. texanus*) across midwestern and eastern USA. In contrast, the other populations across a large and continuous geographical range in Midwestern and eastern USA were not substantially different from one another, showing little genetic differentiation, with some possible exceptions for *C. v. floridanus* and *C. v. texanus*.

Based on genetic structure and geographic ranges, my results suggest that each of *C. v. ridgwayi* and *C. v. floridanus* should be considered as a distinct unit for conservation or management, supporting current subspecies limits. In contrast, *C. v. virginianus*, *C. v. marilandicus*, *C. v. mexicanus,* and *C. v. taylori* may be considered a single management unit because levels of genetic divergence among these putative subspecies were quite low. Among all analyzed subspecies, masked bobwhite has the lowest diversity in all genetic information of both mitochondrial and microsatellite loci. It is not difficult to comprehend that this low genetic diversity is associated with low fitness, reduction in population size, and therefore why masked bobwhite was listed as endangered by the U.S. government and was faced by local extinction. It is highly recommended to set conservation priority to the masked bobwhites as an independent

conservation unit. To better understand genetic structure and diversity of the *C. v. texanus*, and its relationships with other subspecies, it should be analyzed more thoroughly with additional samples and additional populations from Mexico from other purported subspecies.

Genetic information reflects patterns of both historical and contemporary issues. From the genetic data only, therefore, one cannot always infer population processes or dynamics at time frames measured in years to decades, which would be primary issues for the majority of wildlife management (Crandall et al. 2000; Elser et al. 2006; Palsboll et al. 2007). To clarify the subspecies designations as obviously distinct units for conservation and management purpose, it is highly recommended that ecological studies with morphological, demographic, and behavioral information, as well as genetic relationships, should be undertaken and interpreted.

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APPENDIX A

The rQS values for the strict consensus supertree, indicating nodal support (± SE) among the set of source trees. Node numbers refer to Figures 2.2-2.3.

Node number	Clade size	rQS (± SE)	Number of hard matches	Number of hard mismatches	Number of equivocal matches
1	376	1.000 ± 0.051	385	0	0
2	214	0.655 ± 0.042	254	2	129
3	13	0.029 ± 0.012	16	5	364
4	12	–0.003 ± 0.013	12	13	360
5	11	0.031 ± 0.014	20	8	357
6	4	0.036 ± 0.010	15	1	369
7	3	0.005 ± 0.004	2	0	383
8	2	0.005 ± 0.004	2	0	383
9	163	0.566 ± 0.040	225	7	153
10	6	0.047 ± 0.012	20	2	363
11	4	0.026 ± 0.012	15	5	365
12	3	0.003 ± 0.009	6	5	374
13	2	0.005 ± 0.004	2	0	383
14	2	0.005 ± 0.004	2	0	383
15	157	0.301 ± 0.038	162	46	177
16	155	0.262 ± 0.037	154	53	178
17	154	0.262 ± 0.037	154	53	178

18	152	0.262 ± 0.037	154	53	178
19	139	0.330 ± 0.037	162	35	188
20	135	0.358 ± 0.037	169	31	185
21	90	0.081 ± 0.034	103	72	210
22	86	0.049 ± 0.035	101	82	202
23	68	0.023 ± 0.032	78	69	238
24	12	–0.008 ± 0.015	16	19	350
25	5	0.044 ± 0.015	26	9	350
26	3	0.088 ± 0.016	35	1	349
27	2	0.036 ± 0.010	14	0	371
28	2	0.044 ± 0.011	17	0	368
29	7	0.021 ± 0.007	8	0	377
30	3	0.013 ± 0.007	6	1	378
31	2	0.013 ± 0.007	6	1	378
32	4	–0.018 ± 0.007	0	7	378
33	3	–0.010 ± 0.006	1	5	379
34	2	0.003 ± 0.003	1	0	384
35	56	0.187 ± 0.030	102	30	253
36	22	–0.013 ± 0.022	33	38	314
37	20	–0.021 ± 0.021	28	36	321
38	18	0.119 ± 0.021	55	9	321
39	17	0.016 ± 0.020	33	27	325
40	15	0.132 ± 0.021	58	7	320
41	6	–0.049 ± 0.017	13	32	340
42	2	0.000 ± 0.013	13	13	359
43	4	0.049 ± 0.015	26	7	352
44	2	0.052 ± 0.014	24	4	357
45	2	0.047 ± 0.013	22	4	359

46	9	–0.086 ± 0.018	8	41	336
47	6	0.010 ± 0.018	25	21	339
48	2	0.021 ± 0.007	8	0	377
49	4	0.026 ± 0.018	29	19	337
50	3	0.049 ± 0.015	25	6	354
51	2	–0.031 ± 0.013	6	18	361
52	3	0.021 ± 0.016	23	15	347
53	2	0.078 ± 0.014	30	0	355
54	2	0.075 ± 0.014	29	0	356
55	2	0.016 ± 0.007	7	1	377
56	2	0.031 ± 0.009	12	0	373
57	34	0.034 ± 0.020	36	23	326
58	25	0.055 ± 0.018	35	14	336
59	24	0.119 ± 0.019	51	5	329
60	22	0.036 ± 0.019	34	20	331
61	20	0.010 ± 0.018	27	23	335
62	17	0.016 ± 0.018	26	20	339
63	12	0.021 ± 0.014	18	10	357
64	11	0.039 ± 0.010	15	0	370
65	10	0.021 ± 0.010	11	3	371
66	4	–0.005 ± 0.005	1	3	381
67	3	–0.005 ± 0.005	1	3	381
68	2	–0.005 ± 0.005	1	3	381
69	6	0.031 ± 0.009	12	0	373
70	3	0.013 ± 0.007	6	1	378
71	2	0.008 ± 0.006	4	1	380
72	3	–0.003 ± 0.007	3	4	378
73	2	0.016 ± 0.006	6	0	379

74	5	0.008 ± 0.012	13	10	362
75	2	0.013 ± 0.006	5	0	380
76	3	0.034 ± 0.009	13	0	372
77	2	–0.018 ± 0.008	1	8	376
78	3	0.023 ± 0.008	9	0	376
79	2	0.023 ± 0.008	9	0	376
80	2	0.005 ± 0.004	2	0	383
81	2	–0.005 ± 0.006	2	4	379
82	9	–0.003 ± 0.015	17	18	350
83	3	0.010 ± 0.005	4	0	381
84	2	0.000 ± 0.004	1	1	383
85	6	0.013 ± 0.015	18	13	354
86	5	0.044 ± 0.011	17	0	368
87	4	–0.021 ± 0.009	2	10	373
88	3	–0.013 ± 0.009	4	9	372
89	2	–0.008 ± 0.006	1	4	380
90	18	0.086 ± 0.023	54	21	310
91	5	0.125 ± 0.021	56	8	321
92	4	0.078 ± 0.014	30	0	355
93	3	0.031 ± 0.012	16	4	365
94	2	0.003 ± 0.012	12	11	362
95	13	–0.026 ± 0.009	1	11	373
96	12	–0.010 ± 0.008	3	7	375
97	7	0.008 ± 0.009	8	5	372
98	6	0.010 ± 0.009	8	4	373
99	4	0.018 ± 0.009	9	2	374
100	3	0.008 ± 0.009	8	5	372
101	2	–0.003 ± 0.003	0	1	384

102	2	–0.021 ± 0.007	0	8	377
103	5	–0.021 ± 0.009	2	10	373
104	4	0.003 ± 0.005	2	1	382
105	3	0.008 ± 0.005	3	0	382
106	2	0.008 ± 0.005	3	0	382
107	4	–0.018 ± 0.008	1	8	376
108	3	0.008 ± 0.005	3	0	382
109	45	0.078 ± 0.025	60	30	295
110	39	0.029 ± 0.018	30	19	336
111	37	0.021 ± 0.019	30	22	333
112	36	0.021 ± 0.019	30	22	333
113	35	0.044 ± 0.018	33	16	336
114	31	0.047 ± 0.016	28	10	347
115	7	0.075 ± 0.014	29	0	356
116	2	–0.013 ± 0.009	4	9	372
117	5	0.057 ± 0.013	24	2	359
118	4	0.026 ± 0.012	15	5	365
119	3	0.031 ± 0.010	13	1	371
120	2	0.029 ± 0.009	11	0	374
121	24	0.018 ± 0.009	10	3	372
122	23	0.018 ± 0.009	10	3	372
123	16	0.021 ± 0.010	11	3	371
124	12	–0.005 ± 0.009	5	7	373
125	8	–0.010 ± 0.009	4	8	373
126	7	–0.008 ± 0.009	4	7	374
127	6	–0.018 ± 0.007	0	7	378
128	5	–0.018 ± 0.007	0	7	378
129	4	0.026 ± 0.009	11	1	373

130	2	0.010 ± 0.009	8	4	373
131	4	–0.003 ± 0.005	1	2	382
132	3	0.008 ± 0.005	3	0	382
133	7	0.008 ± 0.005	3	0	382
134	6	–0.003 ± 0.005	1	2	382
135	5	–0.003 ± 0.005	1	2	382
136	3	0.003 ± 0.005	2	1	382
137	2	0.008 ± 0.005	3	0	382
138	4	0.005 ± 0.004	2	0	383
139	3	0.005 ± 0.004	2	0	383
140	2	0.003 ± 0.003	1	0	384
141	2	0.003 ± 0.003	1	0	384
142	6	0.094 ± 0.018	41	5	339
143	4	0.016 ± 0.011	12	6	367
144	3	0.000 ± 0.009	6	6	373
145	2	–0.005 ± 0.004	0	2	383
146	13	0.132 ± 0.019	52	1	332
147	12	0.099 ± 0.018	44	6	335
148	7	0.049 ± 0.017	31	12	342
149	6	0.083 ± 0.017	37	5	343
150	4	0.031 ± 0.009	12	0	373
151	2	0.029 ± 0.009	11	0	374
152	2	0.026 ± 0.008	10	0	375
153	5	0.008 ± 0.006	4	1	380
154	4	0.013 ± 0.006	5	0	380
155	3	0.003 ± 0.003	1	0	384
156	2	0.003 ± 0.003	1	0	384
157	2	0.003 ± 0.003	1	0	384

158	2	–0.005 ± 0.004	0	2	383
159	17	0.081 ± 0.017	36	5	344
160	5	0.047 ± 0.012	20	2	363
161	3	0.026 ± 0.012	16	6	363
162	2	0.021 ± 0.008	9	1	375
163	2	0.003 ± 0.003	1	0	384
164	11	0.000 ± 0.013	13	13	359
165	10	0.026 ± 0.008	10	0	375
166	9	0.026 ± 0.008	10	0	375
167	2	0.016 ± 0.006	6	0	379
168	7	0.016 ± 0.006	6	0	379
169	5	0.010 ± 0.007	6	2	377
170	3	0.016 ± 0.006	6	0	379
171	2	0.003 ± 0.006	3	2	380
172	2	0.013 ± 0.007	6	1	378
173	2	0.016 ± 0.006	6	0	379
174	14	0.073 ± 0.014	28	0	357
175	13	–0.008 ± 0.011	8	11	366
176	12	–0.005 ± 0.012	10	12	363
177	7	0.013 ± 0.006	5	0	380
178	6	0.000 ± 0.005	2	2	381
179	2	0.005 ± 0.005	3	1	381
180	4	0.010 ± 0.005	4	0	381
181	3	0.005 ± 0.005	3	1	381
182	2	0.010 ± 0.005	4	0	381
183	5	0.021 ± 0.010	12	4	369
184	2	–0.003 ± 0.007	3	4	378
185	3	0.016 ± 0.007	7	1	377

186	2	0.000 ± 0.007	4	4	377
187	162	0.390 ± 0.034	160	10	215
188	158	0.366 ± 0.031	141	0	244
189	156	0.327 ± 0.031	132	6	247
190	155	0.306 ± 0.031	128	10	247
191	153	0.306 ± 0.031	128	10	247
192	151	0.296 ± 0.031	126	12	247
193	111	0.099 ± 0.022	56	18	311
194	94	0.034 ± 0.021	38	25	322
195	4	–0.044 ± 0.011	0	17	368
196	3	0.034 ± 0.011	15	2	368
197	2	0.008 ± 0.005	3	0	382
198	3	–0.008 ± 0.009	4	7	374
199	2	0.021 ± 0.009	10	2	373
200	41	0.039 ± 0.017	28	13	344
201	35	0.003 ± 0.016	20	19	346
202	34	0.003 ± 0.016	20	19	346
203	29	–0.003 ± 0.015	17	18	350
204	26	0.049 ± 0.015	27	8	350
205	23	0.008 ± 0.015	17	14	354
206	6	0.034 ± 0.011	15	2	368
207	5	0.034 ± 0.011	15	2	368
208	4	0.013 ± 0.008	7	2	376
209	3	0.031 ± 0.009	12	0	373
210	2	0.005 ± 0.004	2	0	383
211	17	–0.016 ± 0.013	10	16	359
212	4	0.031 ± 0.010	14	2	369
213	13	–0.013 ± 0.012	9	14	362

214	2	0.005 ± 0.008	6	4	375
215	11	0.039 ± 0.011	17	2	366
216	10	0.039 ± 0.011	17	2	366
217	9	0.029 ± 0.011	15	4	366
218	8	0.026 ± 0.011	14	4	367
219	5	–0.008 ± 0.009	4	7	374
220	3	–0.005 ± 0.010	7	9	369
221	2	–0.013 ± 0.008	2	7	376
222	2	0.005 ± 0.004	2	0	383
223	3	–0.008 ± 0.007	2	5	378
224	2	–0.005 ± 0.009	5	7	373
225	3	0.036 ± 0.010	14	0	371
226	3	–0.010 ± 0.007	2	6	377
227	2	0.000 ± 0.007	4	4	377
228	5	0.057 ± 0.012	22	0	363
229	3	0.047 ± 0.011	18	0	367
230	2	0.029 ± 0.011	14	3	368
231	2	0.036 ± 0.011	16	2	367
232	6	0.055 ± 0.012	21	0	364
233	4	0.010 ± 0.007	6	2	377
234	3	0.021 ± 0.009	10	2	373
235	2	0.031 ± 0.009	12	0	373
236	2	0.034 ± 0.010	14	1	370
237	2	0.008 ± 0.005	3	0	382
238	4	0.008 ± 0.005	3	0	382
239	2	0.008 ± 0.005	3	0	382
240	8	0.013 ± 0.009	8	3	374
241	2	0.003 ± 0.007	4	3	378

242	6	0.013 ± 0.008	7	2	376
243	5	0.010 ± 0.005	4	0	381
244	4	0.008 ± 0.005	3	0	382
245	3	0.008 ± 0.005	3	0	382
246	2	0.008 ± 0.005	3	0	382
247	2	0.005 ± 0.004	2	0	383
248	2	0.005 ± 0.008	6	4	375
249	8	–0.003 ± 0.010	7	8	370
250	5	–0.016 ± 0.006	0	6	379
251	4	–0.005 ± 0.006	2	4	379
252	3	0.008 ± 0.005	3	0	382
253	2	0.005 ± 0.004	2	0	383
254	2	0.005 ± 0.004	2	0	383
255	2	–0.016 ± 0.006	0	6	379
256	2	0.005 ± 0.004	2	0	383
257	14	0.042 ± 0.015	24	8	353
258	8	0.026 ± 0.012	16	6	363
259	7	0.021 ± 0.011	13	5	367
260	6	0.042 ± 0.010	16	0	369
261	5	0.008 ± 0.005	3	0	382
262	4	0.005 ± 0.004	2	0	383
263	2	0.005 ± 0.004	2	0	383
264	6	0.005 ± 0.006	4	2	379
265	5	0.010 ± 0.005	4	0	381
266	2	0.005 ± 0.004	2	0	383
267	3	0.005 ± 0.004	2	0	383
268	2	0.005 ± 0.004	2	0	383
269	23	0.052 ± 0.015	26	6	353

270	15	0.044 ± 0.013	21	4	360
271	10	0.023 ± 0.010	12	3	370
272	8	–0.013 ± 0.009	3	8	374
273	6	0.000 ± 0.008	5	5	375
274	4	0.008 ± 0.008	6	3	376
275	3	0.000 ± 0.006	3	3	379
276	2	0.010 ± 0.006	5	1	379
277	2	–0.008 ± 0.005	0	3	382
278	2	–0.010 ± 0.005	0	4	381
279	2	–0.013 ± 0.006	0	5	380
280	5	0.023 ± 0.008	9	0	376
281	2	–0.008 ± 0.007	2	5	378
282	3	0.003 ± 0.007	4	3	378
283	2	0.005 ± 0.005	3	1	381
284	8	–0.008 ± 0.012	9	12	364
285	2	0.000 ± 0.010	8	8	369
286	6	0.021 ± 0.007	8	0	377
287	2	0.008 ± 0.005	3	0	382
288	4	0.018 ± 0.007	7	0	378
289	3	0.008 ± 0.005	3	0	382
290	2	0.008 ± 0.005	3	0	382
291	14	0.000 ± 0.015	17	17	351
292	12	0.016 ± 0.014	17	11	357
293	11	0.034 ± 0.014	21	8	356
294	10	0.034 ± 0.014	21	8	356
295	9	0.018 ± 0.014	18	11	356
296	2	0.013 ± 0.006	5	0	380
297	6	0.055 ± 0.012	21	0	364

298	2	0.026 ± 0.008	10	0	375
299	2	0.005 ± 0.006	4	2	379
300	2	0.013 ± 0.006	5	0	380
301	2	0.029 ± 0.009	11	0	374
302	4	–0.044 ± 0.017	13	30	342
303	3	0.091 ± 0.016	36	1	348
304	2	0.005 ± 0.004	2	0	383

APPENDIX B

List of original references containing source trees used in the Galloanserae supergtree construction.

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