SUBSPECIES STATUS OF THE WESTERN YELLOW-BILLED CUCKOO (CUCULIDAE: *COCCYZUS AMERICANUS OCCIDENTALIS*): USING CYTOCHROME B TO ELUCIDATE THE ENIGMA

by

Lindsay L. Farrell

A thesis presented in partial fulfillment of the requirements for the degree of
Master of Science
Graduate Department of Biology, Lakehead University

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ABSTRACT

Subspecies status of the western Yellow-billed Cuckoo (*Coccyzus americanus occidentalis*): Using cytochrome *b* to elucidate the enigma

Masters Degree in Biology, 2006

Lindsay L. Farrell

Department of Biology

Lakehead University

This study documents sequence variation in three mitochondrial DNA (mtDNA) genes in the Yellow-billed Cuckoo (*Coccyzus americanus*) to re-examine the subspecies status of the western population (*C.a. occidentalis*). Described traditionally as occurring from southwestern British Columbia, western Washington, northern Utah, central Colorado, and west Texas south and west to southern Baja California, Sinaloa, and Chihuahua in Mexico (Hughes 1999), the western population was first given the designation of subspecies by Ridgway (1887) based on morphological differences between eastern (*C. a. americanus*) and western forms. Since this initial designation, controversy has ensued over its validity as recent attempts to address the question have produced contradictory results (Spiller 1988; Banks 1988, 1990; Franzreb and Laymon 1993; Pruett et al. 2001). The western

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population has declined rapidly within the last century with an overall range contraction, as their riparian forests have been removed for fuel, agriculture and grazing, and urban development (Laymon and Halterman 1987). Furthermore, lower water tables resulting from river damming and irrigation projects have claimed much of the remaining preferred habitat, leaving only isolated pockets of suitable habitat (Gaines 1974). Consequently, petitions have been submitted to the U.S. Fish and Wildlife Service (USFWS) since 1986 to list the western population under the Endangered Species Act (ESA); unfortunately, they have been largely ignored due to the lack of resolution inherent in the various taxonomic debates. In addition, the USFWS cannot recognize a subspecies or species for listing unless it meets certain mandated criteria for designation as a Distinct Vertebrate Population Segment (DPS). The western Yellow-billed Cuckoo, as currently described, fails to meet these criteria; hence, it is not eligible for conservation and recovery programs mandated for threatened taxa by the ESA. A recent study by Pruett et al. (2001) addressed the subspecies question in the Yellow-billed Cuckoo by analyzing mtDNA sequence variation in cytochrome *b*. They identified four fixed base pair (bp) differences between the eastern and western forms. Although compelling, the work of Pruett et al. had several limitations including small sample size with poor geographic representation of the overall species' distribution, and analysis of only one gene.

I re-examined the subspecies status of the western Yellow-billed Cuckoo by analyzing cytochrome *b*, ND2 (NADH dehydrogenase subunit 2) and ND6 (subunit 6) sequences with a large geographic distribution of samples representative of

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overall species range with focused sampling effort on the zones of contact between the two populations. Two data sets were compiled for this study: one set that consisted of only my data and a second set that incorporated Pruett et al. (2001) sequences. Maximum likelihood trees and haplotype diagrams were generated using both data sets, each being used in two separate analyses, with and without third codon positions. Statistical analysis were conducted prior to tree reconstruction in order to determine the appropriate model of nucleotide substitution under the Akike Information Criterion, and to estimate the gamma distribution parameter of rate heterogeneity and proportion of invariant sites.

In all data sets, the trees and haplotype diagrams revealed only limited genetic divergence between eastern and western populations of Yellow-billed Cuckoo. For example, the gene sequences of ND2 and ND6 revealed no variation between eastern and western populations. Some localized structure was evident in trees and haplotype diagrams reconstructed from cytochrome *b* sequences; although, the four fixed base pair differences purportedly discovered — and used to distinguish the two subspecies — by Pruett et al. (2001) were not present in my data set. I suggest that this alleged variation is merely the result of damage to the DNA template caused by the preservative DMSO (dimethyl sulfoxide) and improper storage conditions of their samples, not phylogenetic signal as their study implied.

Although I cannot report genetic separation between eastern and western populations of Yellow-billed Cuckoo based on my analysis, my results document a substantial amount of haplotype variation within the western samples, which may be indicative of local inbreeding and some degree of substructuring among these

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declining populations. Nonetheless, it is evident that cytochrome *b*, ND2 and ND6 are not sufficiently variable to detect genetic differentiation within this species. This is not to suggest that the eastern and western populations of Yellow-billed Cuckoo are not valid subspecies, simply that the genetic markers used are not suitable genes to distinguish the two forms. Future studies using more-rapidly evolving genes, such as the mitochondrial control region, may prove more rewarding in this regard.

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Introduction

Western Yellow-billed Cuckoos (*Coccyzus americanus occidentalis*) are quickly vanishing from their native range and soon may not be heard making their characteristic call before the rain. Once considered common in river bottoms throughout the western United States and southern British Columbia, numbers of cuckoos have plummeted— concurrent with an overall range contraction — with the destruction of their riparian forests for fuel, agriculture and grazing, and urban development (Laymon and Halterman 1987). Furthermore, lower water tables resulting from river damming and irrigation projects have claimed much of the remaining preferred habitat (Gaines 1974). Chemical spraying of farmlands and forests have also caused widespread pesticide poisoning. The impact of pesticide use on cuckoo reproduction is not well understood; however, eggshell thinning associated with organochlorine pesticide (e.g., DDT) accumulation in adipose tissue (Grocki and Johnston 1974) — similar to that which caused endangerment of Peregrine Falcons (*Falco peregrinus*) — has been reported in both Yellow-billed and Black-billed cuckoos (*Coccyzus erythropthalmus*; Laymon 1980; Laymon and Halterman 1987).

The western Yellow-billed Cuckoo has been traditionally described as occurring from southwest British Columbia, western Washington, northern Utah, central Colorado, and west Texas south and west to southern Baja California, Sinaloa, and Chihuahua in Mexico (Hughes 1999; Figure 1). The eastern nominate population (*Coccyzus americanus americanus*) occupies the remainder of the species' range in North America, which extends to eastern Mexico and the Greater

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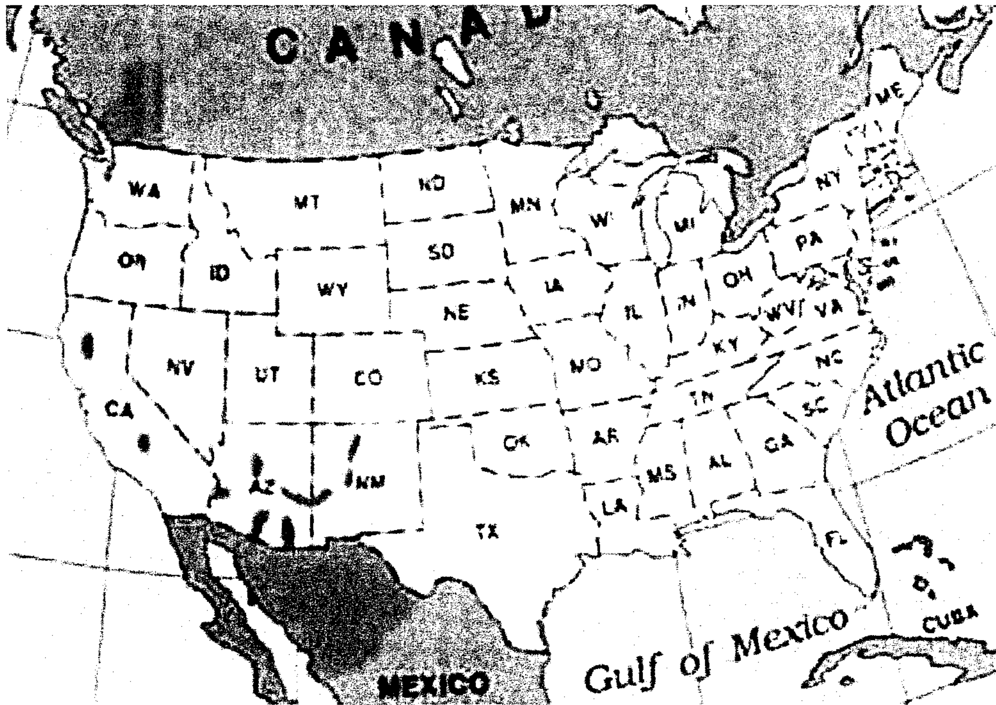
Figure 1. Western range of the Yellow-billed Cuckoo (*Coccyzus americanus*). Pink shaded area represents historic range, whereas red shaded area represents estimated current range (after Laymon and Halterman 1991).



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Antilles (Hughes 1999; Figure 2). Oberholser (1974) believed that the boundary between the two forms occurred along the Pecos River in west Texas, and this is now considered the zone of secondary contact between the two forms.

Eastern Yellow-billed Cuckoos breed in a wide range of habitats including deciduous forests and parks. The western population, however, requires large stands of streamside, or riparian, forests characterized by cottonwoods and willows for nesting (Ehrlich et al. 1988). However, less than one percent of the original riparian forest remains in California, and what is left is surrounded by agricultural croplands and large sections of stripped land used for grazing cattle (Laymon and Halterman 1987). Consequently western cuckoos, once considered to be common breeders, have declined throughout the state to a current population of less than 50 pairs (Gaines and Laymon 1984; Laymon and Halterman 1991). The Sacramento River Valley in California, historically a prime breeding location, now supports only a few breeding pairs in isolated pockets of remaining nesting habitat. It is now extremely rare elsewhere in the western United States and is already extirpated from British Columbia, Washington, Oregon, and Nevada. There is currently no federal protection for either full species or western populations (Hughes 1999).

The apparently imminent demise of the western Yellow-billed Cuckoo has caused ornithologists to reconsider its subspecific status, particularly in light of recent petitions to warrant listing the western population as a protected taxon under the Endangered Species Act (ESA) by the U.S. Fish and Wildlife Service (USFWS). Unfortunately, the USFWS cannot recognize a subspecies or species for listing unless it meets certain mandated criteria for designation as a Distinct Vertebrate

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Figure 2. Eastern range of Yellow-billed Cuckoo (*Coccyzus americanus*) shaded in green (after Cornell Lab of Ornithology 2005).

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Population Segment (DPS). The western Yellow-billed Cuckoo is not currently designated as a DPS; hence, it is not eligible for conservation and recovery programs mandated for threatened taxa by the ESA.

The western Yellow-billed Cuckoo was once considered to be part of the eastern population. However, Ridgway (1887) first proposed the western form as a separate subspecies because he considered them to be "*larger with proportionately larger and stouter bills*" than the eastern form. Accordingly, he assigned Yellow-billed Cuckoos that occurred from extreme west Texas and north along the Pacific Coast to the western subspecies and other North American populations to the eastern subspecies. The western subspecies also included birds from the Great Basin portions of Colorado and Wyoming, west and north to the Pacific Coast and southwestern British Columbia (Ridgway 1887). The two subspecies were generally accepted in many ornithological publications (Peters 1940; American Ornithologists' Union 1957; Wetmore 1968; Oberholser and Kincaid 1974); although, others have questioned the validity of such separation (Todd and Carriker 1922; Swarth 1929; Van Tyne and Sutton 1937; Bent 1940; Mees 1970; Monson and Phillips 1981; Banks 1988, 1990).

Furthermore, the two subspecies differ in morphology, ecology, and behaviour. Western Yellow-billed Cuckoos are somewhat grayer dorsally than their eastern counterparts, most notably on the crown, and have an orange-yellow, rather than yellow, lower mandible. They are also purportedly larger than eastern birds, particularly with respect to wing and bill lengths (Hughes 1999). Unfortunately, recent attempts to identify the subspecies based on morphology have yielded

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contradictory results (Spiller 1988; Banks 1988, 1990; Franzreb and Laymon 1993).

Tape recordings collected by Franzreb and Laymon (1993), however, support differences in vocalizations between eastern and western birds. These authors suggest that the species' characteristic *Kowlp* Call — a frequently-heard vocalization given by the male to delineate territory — is more feeble in the western Yellow-billed Cuckoo than in the eastern form, and resembles the territorial call of the Black-bill Cuckoo (*Coccyzus erythropthalmus*; Hughes 1999). In addition, Franzreb and Laymon (1993) indicate a difference in juvenal plumage; nestlings in California have entirely-black bills for at least three weeks before leaving the nest, whereas, juveniles in the east have yellow bills during this period of time.

There also appears to be differences in breeding phenology between the two cuckoo populations; egg records indicate that eastern birds begin breeding considerably earlier than their western counterparts. For example, in eastern Texas eggs have been noted as early as 24 March and as late as 30 June (n = 34), with the most occurring between 6 May and 6 June (n = 26). In Illinois, eggs were reported from 20 May to 19 July (n = 39), with about 50 percent occurring from 4 to 26 June. In contrast, eggs in California have been observed from 15 May to 20 August (n = 55), with 51 percent occurring from 17 June to 10 July. Egg dates in Arizona range from 28 June to 24 August (n = 13; Bent 1940). Clearly breeding in eastern cuckoos begins earlier, regardless of latitude, with most clutches laid two weeks to three months before those of western birds (Franzreb and Laymon 1993).

Furthermore, studies demonstrate differences in timing and routes of migration, and wintering distribution of eastern and western Yellow-billed Cuckoos.

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Eastern birds winter east of the Andes Mountains in South America from Venezuela and Bolivia south to northern Argentina. They migrate north in spring through Central America and the West Indies, arriving on North American breeding grounds in late April (AOU 1957; Brewer 1991; Haverschmidt and Mees 1994; Hughes 1999). In contrast, the western population winters in western South America from Colombia south to northwestern Argentina. They travel north via the Pacific Slope of Mexico and Central America and arrive on the breeding grounds no earlier than late May, four to eight weeks later than eastern cuckoos breeding at the same latitude (Wetmore 1968; American Ornithologists' Union 1983; Franzreb and Laymon 1993; Laymon 1998; Hughes 1999; Figure 3). These fundamental differences in wintering distribution, and route and chronology of migration support a distinct and ancient divergence between eastern and western Yellow-billed Cuckoo populations (Hughes 1999).

First Petition to list the Western Subspecies

The noticeably rapid decline of the western Yellow-billed Cuckoo in the 1970s and 1980s have led to some efforts to save this potential subspecies from extinction. In 1986, the Nature Conservatory initiated reforestation programs at suitable sites in the Kern River Preserve in Weldon, California in order to attract breeding pairs. However, without state or federal legislation to protect the species, long-term efforts such as this could be both futile and unsustainable financially. Consequently in 1986, a petition was submitted by T. Manolis (Acting President of the Western Field Ornithologists) and cosigned by representatives of the Animal Protection Institute,

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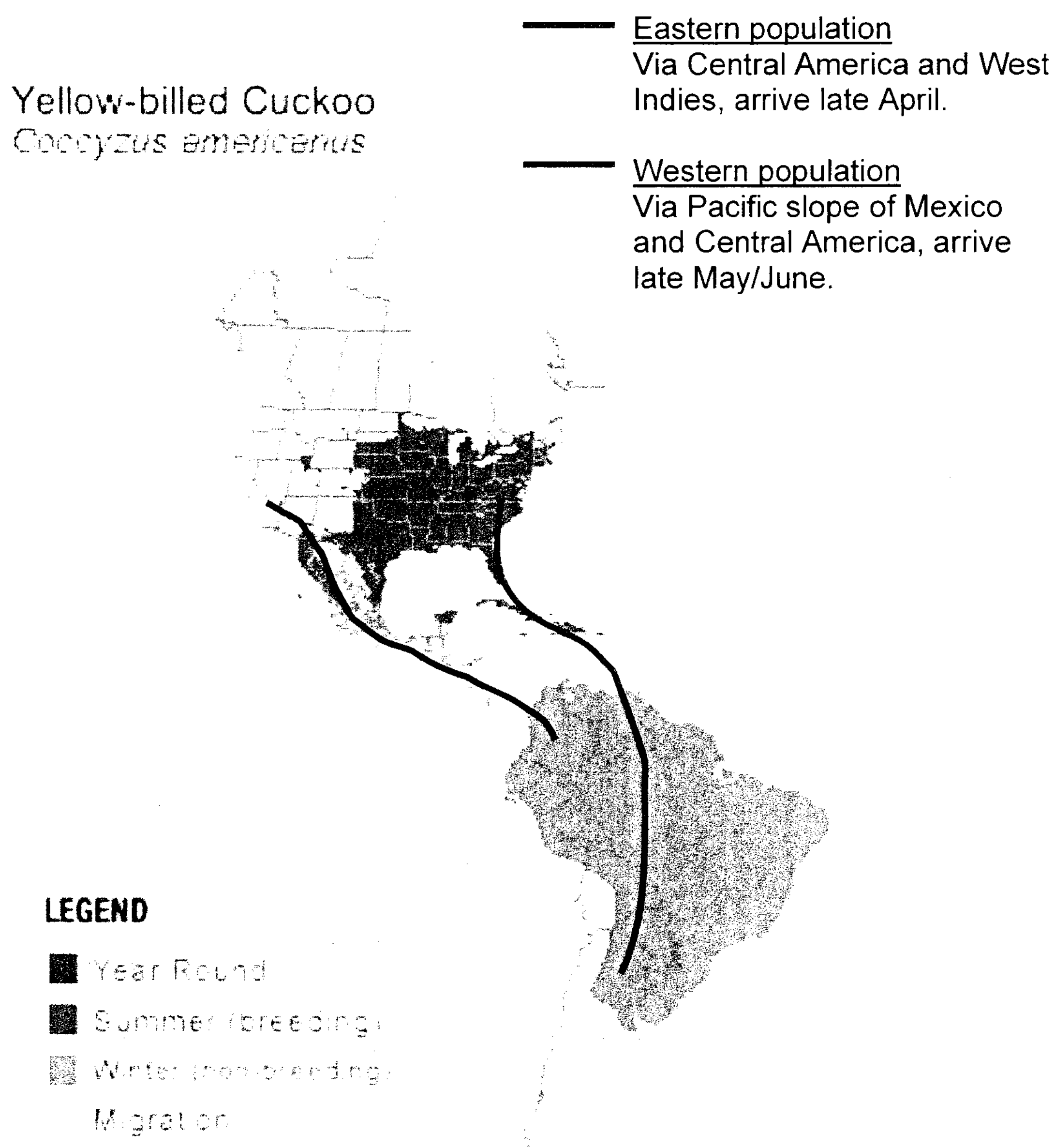
Figure 3. Migratory routes of eastern and western populations of Yellow-billed Cuckoo (*Coccyzus americanus*) (AOU 1957; Wetmore 1968; AOU 1983; Brewer 1991; Franzreb and Laymon 1993; Haverschmidt and Mees 1994; Laymon 1998; Hughes 1999). The eastern population migrates via Central America and the West Indies to Central America, whereas the western population is thought to migrate via the Pacific slope of Mexico and Central America (Figure courtesy of Lab of Cornell Ornithology).



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Defenders of Wildlife, Sacramento River Preservation Trust, Friends of the River Planning and Conservation League, Davis Audubon Society, Sacramento Audubon Society, and Sierra Club to USFWS to list the western Yellow-billed Cuckoo under the Endangered Species Act in the states of California, Washington, Oregon, Idaho, and Nevada (Manolis et al. 1986). In response to this submission, the Federal Register published a 12-month finding (52 FR 2239) stating that the petition presented substantial information to warrant listing (USFWS 2001).

In 1988, however, the Federal Register published a second finding (53 FR 52746) stating that the petitioned action was not warranted based on the findings of Banks (1988). In his study, Banks evaluated nearly 700 museum specimens of adult cuckoos using three morphological characteristics (bill length, depth of upper mandible, and wing length) and plumage colour comparisons. Banks concluded that his data failed to demonstrate consistent morphological differences between eastern and western birds, and he could not justify their separation into two subspecies. He added that there remained the possibility of an isolated breeding population of Yellow-billed Cuckoos within the western region, but that a subspecific status for *C. a. occidentalis* was not defensible.

Shortly after publication of Banks (1988), Spiller (1988) found statistical errors in Banks' study upon re-examination of the same data. Spiller showed that the morphological differences between eastern and western subspecies were highly statistically significant ($p<0.001$; USFWS 2001). Banks (1990) later acknowledged the statistical error but, in spite of this, maintained that the subspecies separation was not warranted. Furthermore, he recommended that the USFWS down-list

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western Yellow-billed Cuckoos, thereby, denying a severely declining species any means of federal protection.

Consequently, the western Yellow-billed Cuckoo population took a severe blow in 1990 when it was down-listed to Category 3B (former federal candidate for listing) from its previous 1986 Category 2 (candidate for listing) designation by the USFWS (Hughes 1999). Since that time, a few authors have produced additional papers refuting Banks' claim of a monotypic species (Laymon and Halterman 1991; Franzreb and Laymon 1993; Pruett et al. 2001) that, by definition, is not divisible into recognizable subspecies. For example, Franzreb and Laymon (1993) reassessed Banks' original data with the addition of a fourth character (tail length). Similar to Spiller (1988), Franzreb and Laymon concluded that the status of two subspecies be recognized based on statistically significant differences between eastern and western birds ($p<0.05$) in these morphological characters. More importantly, these authors described differences in behaviour, such as breeding habits and chronology, vocalization, and migration. They recommended that the subspecies status of the western population be retained until further analysis of geographic variation in morphology, behaviour, and genetics is performed (Franzreb and Laymon 1993).

Second Petition to List Western Subspecies

In February of 1998, the USFWS received a petition from the Southwest Center of Biological Diversity in Tucson, Arizona on behalf of 22 groups to list the western population of Yellow-billed Cuckoo under the Endangered Species Act. The document recommended additionally that streams and rivers in the western United

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States from Washington State to Texas be designated as *critical habitat* for the species. This petition included supporting information on the species relating to taxonomy, ecology, historic and present distribution, habitat requirements, current status, and threats (USFWS 2001). It presented substantial scientific and commercial information to support listing the western Yellow-billed Cuckoo, but also recommended that further investigation be undertaken to determine the taxonomic validity of the western subspecies, and whether designating it as a Distinct Vertebrate Population Segment (DPS) was warranted.

The Federal Register subsequently released the findings of their 90-day (65 FR 8104) review of evidence (USFWS 2001) on 17 February 2000. This stated that the American Ornithologists' Union Committee on Classification and Nomenclature — which consisted of only six North American ornithologists — agreed with Banks' original 1988 decision that the Yellow-billed Cuckoo should be considered a monotypic species with no distinct well defined subspecies, therefore, it did not meet the criteria of a DPS under the USFWS (USFWS 2001).

As a result of this negative response, the Center for Biological Diversity — on behalf of the Maricopa Audubon Society, Oregon Natural Desert Association, Wildlife Damage Review, Sky Island Alliance, Biodiversity Legal Foundation, Wetlands Actions Network, Klamath-Siskiyou Wildlands Center, and Oregon Natural Resources Council (ONRC) Fund — filed a 60-day notice of intent to sue based on the negative 90-day finding. They stated that the Service's finding was "*arbitrary and capricious*" and "*not based on the best available scientific and commercial information available*" as was required by the Endangered Species Act (Suckling

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2000). They threatened that suit would be filed within 60 days unless the Service either (1) published a new finding in the Federal Register concluding that it possessed substantial scientific information indicating the Yellow-billed Cuckoo (as a species) may be threatened or endangered in a significant portion of its range, or (2) responded in writing, indicating that the 90-day finding was not intended to preclude a full review of the possibility of listing the species based on imperilment in a significant portion of its range, that such a review would be part of the Yellow-billed Cuckoo Status Review and, further, that the 12-month finding would include a decision on whether the Yellow-billed Cuckoo is threatened or endangered in a significant portion of its range (Suckling 2000).

In response to these threats, the USFWS and the United States Geological Survey (USGS) funded a genetic study in January 2000 to determine whether the western form of Yellow-billed Cuckoo was a valid subspecies or a Distinct Vertebrate Population Segment, in which case a listing proposal could be supported. Under the Endangered Species Act, the USFWS must consider listing any species, subspecies, or DPS vertebrate, if there is sufficient information to indicate that such an action is warranted (USFWS 2001). The USFWS uses two elements to assess whether a population segment under consideration for listing may be recognized as a DPS: (1) the degree of discreteness of the population segment from the rest of the taxon, and (2) its significance as a unique unit to the taxa to which it belongs (USFWS 2001). However, in the case of the Yellow-billed Cuckoo, controversy still surrounded the status of the western subspecies. If it were reevaluated by the USFWS and gained recognition as a DPS, it would then qualify for protection under

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the Endangered Species Act.

From a total of five proposals received, funding was given to Robert Fleischer of the Smithsonian Institution. On 24 April 2001, he presented his findings concluding that, based on the analysis of two mitochondrial genes (ATPase8 and Control Region), no valid subspecies exist for the Yellow-billed Cuckoo (USFWS 2001). When Fleischer's study was peer reviewed in January of 2001, it met with varying degrees of acceptance. Three reviewers agreed with his conclusions that there is no substantial genetic differentiation between eastern and western individuals. However, three reviewers concluded that there was evidence to suggest a recent range expansion, and three others suggested that his particular use of mtDNA might be inadequate and other genetic markers should be considered (K. Suckling, *pers. comm.*). In summary, Fleischer's study suffered significantly from having used museum specimen toe pads as a source of DNA for substantial portion of the study. This potentially degraded source of DNA generated sequences only 422 and 314 base pairs long for control region and ATPase8, respectively. Furthermore, ATPase 8 has been shown to be problematic in phylogenetic inference because what appears to be phylogenetic signal is often merely an expression of haplotype frequency differences, which are inadequate to resolve evolutionary relationships (Taylor 2001).

In July of 2001, at the end of the open comment period, the USFWS Federal Register published a decision to again delay federal protection for the western population of Yellow-billed Cuckoos under the Endangered Species Act. Federal Register "Notice One-year Petition Finding" (66 FR 38611) declared that the species

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"*warrants*" listing as threatened, but that the USFWS would not issue a proposal at this time, due to other "*higher priority*" listings (Suckling 2001). Accordingly, the western Yellow-billed Cuckoo was placed on the warranted-but-precluded list.

Unfortunately, species on the warranted-but-precluded list receive no legal protection, nor is there a time limit as to how long they remain on the list. Of 24 species currently on the warranted-but-precluded, the average length of time since they were petitioned for listing is 10 years, including a 19-year delay for the sheath-tailed bat (*Emballonura semicaudata*), a 15-year delay for six species of New Mexico spring snails (*Juturnia kosteri, Pseudotryonia alamosae, Assiminea pecos, Pyrgulopsis chupaderae, P. neomexicana*, and *P. roswellensis*), and 13-year delays for Columbia and Oregon spotted frogs (*Rana luteiventris and R. pretiosa*; Suckling 2001). Federal courts have repeatedly ruled that the USFWS has illegally stuck imperiled species on the warranted-but-precluded list as delay tactics to avoid angering powerful industry lobbyists (Suckling 2001). The placement of the bull trout (*Salvelinus confluentus*) and Canada lynx (*Lynx canadensis*) on the warranted-but-precluded list were both overturned by federal judges. The Center for Biological Diversity planned on filing suit over the newest delay for the protection of the western Yellow-billed Cuckoo in the hope that the current ruling would also be overturned (Suckling 2001), but they have not as yet taken any legal action on their behalf.

Recent Studies

In 2001, Pruett et al. conducted the first genetic study on the Yellow-billed Cuckoo

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and provided evidence to separate the species into two distinct subspecies — eastern *C.a. americanus* and western *C. a. occidentalis.* They found that the haplotypes of the eastern and western forms differed by four fixed base changes present in a 978 base pair (bp) portion of the cytochrome *b* gene. Due to this multiple fixed base change difference in mitochondrial DNA (mtDNA), they further suggested that the eastern and western subspecies have not shared a common ancestor for hundreds of thousands of years. Contrary to Banks (1988), this finding recognized the western form as a subspecies — a distinct vertebrate population segment — under the Endangered Species Act. Pruett et al. (2001) was seminal among efforts to validate the western subspecies Yellow-billed Cuckoo, however, it had several limitations.

For example, Pruett et al. (2001) used only ten individuals. Five specimens were designated as belonging to the western subspecies; two were from Alaska and three from New Mexico. This left a substantial portion of the overall species distribution unsampled, including the primary range of the western population (i.e., California, Arizona, and Texas; Figure 1). There was also the possibility that the Alaskan individuals could be vagrants from the eastern population. Historically, western Yellow-billed Cuckoos had a much smaller range than their eastern counterparts, which did not include Alaska. Moreover, the subspecies was extirpated from the most northern part of their distribution in British Columbia during the late 1920s (Hughes 1999). Pruett et al. (2001) also analyzed two individuals from Minnesota, one from Vermont, and two from Vera Cruz, Mexico; nonetheless, the majority of the species range was left unsampled.

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In addition to the small sampling distribution used in Pruett et al. (2001), most samples in the data set were either the property of (or prepared at) the University of Alaska Museum (UAM). This could be problematic due to numerous different methods in which museums store and preserve tissue samples. Sequence variation in polymerase chain reaction (PCR) products from museum specimens can be incorrect due to regular DNA polymerase errors, as well as damage in the DNA template (Hansen et al. 2002). For example, it is known that nucleic acids undergo spontaneous decomposition in solution (Lindahl 1993) and are particularly prone to oxidative damage (Dizdaroglu et al. 2002). Thus, preserved tissue samples may exhibit DNA damage — beyond the initial damage caused by decay — during storage in either a buffer solution or other preservative under conducive conditions. Therefore, it is prudent to compile samples granted from several different museums or tissue collections to avoid widespread errors due to improper storage conditions, if a data set is to be constructed primarily of frozen or preserved tissue samples.

Furthermore, Pruett et al. (2001) examined a 978 bp sequence fragment of mtDNA that included part the cytochrome *b* gene and found that all haplotype variation was only present in the latter part of their sequence. The primer sets used in their study ended prematurely; it was possible that further variation could be present near the end of cytochrome *b* that was not sequenced. The study would also have benefited with the addition of more sequence data, including other mitochondrial genes. Cytochrome *b* is considered a relatively slow-evolving gene that may have limited utility at the subspecies level, lacking the specificity to discern relatively recent population-level genetic differentiation among taxa or to account for

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limited genetic structure that may be present among individuals in the areas of overlap between the two recognized population ranges.

Pruett et al. (2001) identified four fixed base pair differences within cytochrome *b* between haplotypes of the two subspecies, which separated the eastern population from western population with sequence divergences ranging from 0.41–0.92%. Based on Shields and Wilson (1987) — who estimated a rate of cytochrome *b* evolution in birds approximately two percent divergence per million years — Pruett at al. suggested that the two subspecies diverged approximately 205,000–465,000 years ago. However, recent rate estimates of cytochrome *b* evolution in cuckoos indicate that Pruett et al. may be underestimating the rate of divergence by a factor of four, which could indicate a divergence between the eastern and western populations by a million years or more (Hughes, *ms* in review).

Finally, Pruett et al. (2001) was unable to address the question of clinal variation in the Yellow-billed Cuckoo due to their limited sample distribution and the nature of their cytochrome *b* sequence findings, which only delineated extremes in the east and west populations. There have been suggestions that the observable differences between the two subspecies are simply a clinal shift in morphology across the species range, not the evolution of distinct lineages. Consequently, many who oppose giving a subspecific status to the western populations claim that the Yellow-billed Cuckoo is merely a monotypic species with variation defined by intermediates or isolated breeding populations (Mees 1970; Banks 1988,1990). This view, however, clearly ignores many of the behavioral and ecological differences noted between the two forms and could easily be a legal loophole that will eventually

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permit the eradication of the western form.

Mitochondrial DNA is characterized by relatively rapid rates of evolution, as compared to nuclear DNA, and an uncomplicated maternal mode of inheritance. Therefore, it may offer a sensitive molecular probe that is appropriate to elucidate intraspecific evolutionary processes (Ball and Avise 1992). In my study, I sequenced three target regions of the mitochondrial genome — cytochrome *b*, ND2, and ND6 (NADH dehydrogenase subunit 2 and 6) — to re-examine, and possibly augment, the findings of Pruett et al. (2001). Taking other limitations of Pruett's study into consideration, I also chose to increase the number of samples from 10 to 31; thus, representing a larger geographic distribution across the species range, and focusing on the overlapping zones of contact between the two populations. Given that nucleotide sequences retain a record of an organism's evolutionary history, phylogeographic analyses can offer a useful reflection of the histories of populations (Friesen et al. 1996).

Despite the evidence that supports the subspecies designation both traditionally (Ridgway 1887; Peters 1940; Wetmore 1968; Oberholser and Kincaid 1974) and in recent years (Laymon and Halterman 1991; Franzreb and Laymon 1993, Pruett et al. 2001), the western Yellow-billed Cuckoo is still not formally recognized by the American Ornithologists' Union, the primary North American authoritative body for taxonomic nomenclature of birds. The fundamental intention of my study was to provide additional insights into the question of whether or not a subspecies status for the western Yellow-billed Cuckoo was warranted.

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Materials and Methods

The Yellow-billed Cuckoo (*Coccyzus americanus*) is typically classified as a monotypic species (Todd and Carriker 1922; Swarth 1929; Van Tyne and Sutton 1937; Bent 1940; Mees 1970; Monson and Phillips 1981; Banks 1988,1990; American Ornithologists' Union 1983, 1998), despite the fact that a distinct western subspecies (*C. a. occidentalis*) has been recognized by some authorities for nearly 120 years (Ridgway 1887; Peters 1940; American Ornithologists' Union 1957; Wetmore 1968; Oberholser and Kincaid 1974; Gaines and Laymon 1984; Laymon and Halterman 1991; Franzreb and Laymon 1993). The disagreement on the validity of the western subspecies of Yellow-billed Cuckoo, stems from conflicting data in studies that have attempted to address this question (Banks 1988, 1990; Spiller 1988; Franzreb and Laymon 1993; Pruett et al. 2001). Most recently, Pruett et al. (2001) suggested that eastern and western populations may be separated by four fixed base pair variations present within the cytochrome *b* gene. Consequently, I chose to re-examine the sequence variation in cytochrome *b* — in addition to two other genes that were not studied — with a larger sampling effort than was previously undertaken.

Tissue samples

My study includes the largest data set — in terms of both sequence length and number of taxa included — ever assembled for the Yellow-billed Cuckoo. I determined gene sequences in a total of 31 individuals derived from 21 tissues and

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10 blood samples granted through loan from museums and other researchers in the field. The samples were collected over a wide geographic area spanning a substantial portion of the species' range (Table 1; see Figure 4) including the ostensible areas of overlap between the two subspecies.

Samples were designated as being either "eastern" or "western" based on where they were located in relation to the historical and current range of the species and subspecies (Hughes 1999). Thus, samples in California, Arizona, New Mexico, and west Texas were considered western and all other samples situated north and east of west Texas were attributed to the eastern population. Accordingly, 19 samples were designated as western and 16 samples as eastern. Franzreb and Laymon (1993) conducted a morphometric analysis of 41 Yellow-billed Cuckoo specimens and concluded that the purported zone of overlap between the eastern and western subspecies occurs in western Texas, eastern New Mexico, and the states of Chihuahua and Coahuila in adjacent Mexico. I paid special attention to these potential zones of contact between populations to account for possible intermediates, thereby, addressing the suggestion of clinal variation within the species. To increase the sample size, I also used published sequences from Genbank, including two from Mexico (AY46908, AY46909), three from New Mexico (AY46905, AY46906, AY46907), two from Alaska (AF249268, AF249269), two from Minnesota (AF249270, AF249271), and one from Vermont (AY469190). These final five sequences (Alaska, Minnesota, and Vermont) were posted to Genbank by Pruett et al., being derived from their study. A cytochrome *b* sequence from Laurel, Maryland (MKP 881) was also included in my analysis (Hughes 2007).

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Table 1. Locality information of all Yellow-billed Cuckoo (*Coccyzus americanus*) tissue and blood samples used in this study corresponding to continental distribution map (Figure 4).

Map#	State	Accession	Map#	State/Province	Accession
1	Alaska	UAM CLD 399	8	Texas	NK 11964
2	California	AMNH 381	9	Texas	LSUMZ B-37016
3	California	MH119-207555	10	Texas	LSUMZ B-23134
	California	MH119-7040	11	Texas	LSUMZ B-21785
	California	MH119-207553	12	Louisiana	UWBM 615
4	Arizona	MH119-207576	13	Florida	UFNUMB 44090
	Arizona	MH119-20742	14	Florida	UFNUMB 44087
	Arizona	MH119-207535	15	North Carolina	UWBM 2510
	Arizona	MH1212-13712	17	New York	AMNH 619
	Arizona	MH1212-13715	18	Massachusetts	UWBM 68159
	Arizona	MH1212-13719	20	Wisconsin	FMNH 441576
	Arizona	MH119-207594	21	Illinois	FMNH 363714
	Arizona	MH119-207596	22	Iowa	FMNH 429371
5	New Mexico	NK11992	23	Kansas	MBR 2690
6	New Mexico	NK 103366	24	Kansas	MBR 27
7	New Mexico	NK 116168	-	Alberta	RAM Z.01.3.1

Specimen numbers refer to source collections: UAM, University of Alaska Museum, Fairbanks; AMNH, American Museum of Natural History, New York; NK, University of New Mexico, Albuquerque; FMNH, Field Museum of Natural History, Chicago; MBR, University of Kansas Natural History Museum, Lawrence; UF, Florida Museum of Natural History, Gainesville; UWBM, University of Washington Burke Museum of Natural History, Seattle; ANSP, Academy of Natural Sciences of Philadelphia; RAM, Royal Alberta Museum, Edmonton; LSUMZ, Louisiana State University Museum of Natural History, Baton Rouge; MH, Murrelet Halterman, University of Nevada, Reno.

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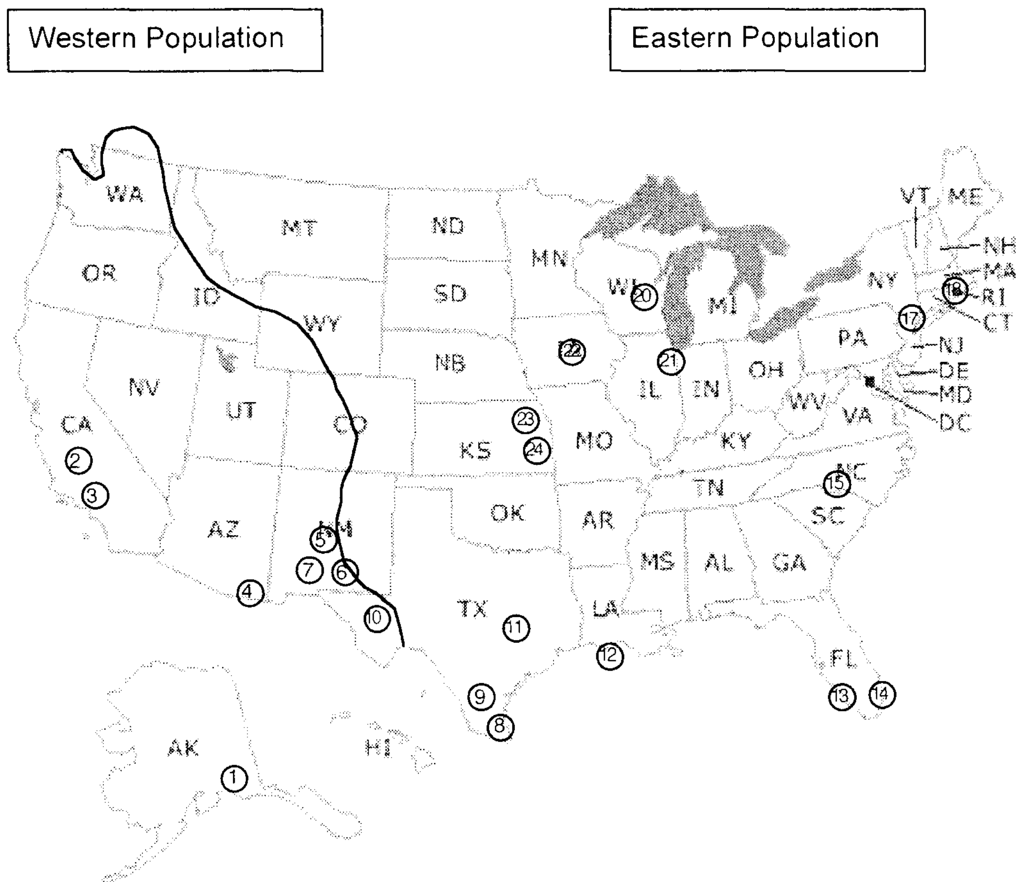
Figure 4. Continental distribution of Yellow-billed Cuckoo (*Coccyzus americanus*) tissue samples used in this study. Historical range defined by black line. All samples located west of historical boundary are attributed to the western population and all samples located east and north of the boundary belong to the eastern population. Numbers correspond to individual samples used in this study. One additional sample, which is not included on this map, was granted from the Royal Alberta Museum in Edmonton, Alberta, Canada (RAM Z01.3.1).



Historical range of the western Yellow-billed Cuckoo (after Laymon and Halterman 1991).

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Twenty-five samples comprised fresh liver and pectoral muscle tissue from specimens preserved for molecular study in 100% EtOH or HCl Tris lysis buffer. Ten blood samples were preserved on FTA® cards (Fluorescent Treponema Absorption cards), where blood had been specifically collected by another researcher for microsatellite and population analysis from two breeding populations in California and Arizona. Approximately 2 mm^2 of tissue was cut out by scalpel and digested with 2 μl of proteinase k, 290 μl of TNE, 40 μl 20% SDS, 40 μl DTT (0.39 M) and 28 μl H_20. Digestions proceeded at 56°C with agitation overnight. The following day, 40 μl of 10% (w/v) Sodium acetate was added, and the solution was vortexed for 1 minute. Subsequently, 1,100 μl of cold EtOH (2.5 x vol) was added and placed on ice for 30 minutes. The product was centrifuged for 5 minutes at 1,300 rpm, after which the supernatant was discarded and remaining precipitate air-dried at room temperature for 30 minutes. It was then resuspended in 150 μl of H_20, and incubated at 37°C for 15 minutes. For some samples, DNA had to be further purified by extra centrifugation at 1,300 rpm for 4 minutes prior to adding sodium acetate due to an excess of undigested protein secondary structure that was left behind by the proteinase K. This was more common in the pectoral muscle tissue samples than liver samples, where pieces of fibrous striated muscle were often the DNA source.

Sequencing Analysis

Three mitochondrial genes were used in my study. First, I sequenced cytochrome *b*

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in order to re-examine the findings of Pruett at al. (2001). However, unlike their study I sequenced beyond the end of the gene, thus, providing a potentially more informative gene fragment on which to base my conclusions.

Although the entire mtDNA sequence is known for many birds (Desjardins and Morais 1990), most studies that use mtDNA sequence variation in phylogenetic or population analyses focus on a narrow set of markers such as the control region and, most frequently, cytochrome *b* (Jones and Gibbs, 1997). However, little information is available to describe levels of variation in other mtDNA genes in birds. One such gene, which is rarely used, but which may prove useful for reconstructing phylogenies of closely related species and performing intraspecific phylogeographic analyses, is the NADH dehydrogenase subunit 6 gene (ND6) (Jones and Gibbs 1997). Hence, I also chose to sequence ND6 in hope that it might provide another level of resolution that is not accomplished using cytochrome *b*.

I also sequenced NADH dehydrogenase subunit 2 (ND2). ND2 is a useful gene for subspecies level investigations used widely in avian studies because the complete gene can be amplified in either one or two fragments with primers that have worked well on most bird species (Greenberg et al. 1998; Bates et al. 1999; Johnson and Lanyon 1999). Furthermore, ND2 is the third most variable gene — the control region and ATPase8 being the first and second most variable, respectively. It was the aim of this study that using three target regions, rather than only one as in Pruett et al. (2001), would provide information at differing levels of resolution, enabling a more robust phylogenetic analysis.

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Primer design

Cuckoos possess a novel mitochondrial gene order in which the control region has been translocated in the 3' to 5' direction. The control region is flanked by cytochrome *b* and ND6, rather than being positioned between ND6 and 12S (Kvist 2000; Figure 5). Consequently, I was required to design several new primers, and modify existing primers, in keeping with gene order considerations (Table 2).

Two previously published primers (Sorenson et al. 1999; Sheldon et al. 2005) were used unmodified to amplify the first region of cytochrome *b*. However, amplifying the entire cytochrome *b* gene in one fragment was found to be problematic, due to primer specificity and large amplicon size. Hence, I designed two internal cytochrome *b* primers (CYTB1, CYTB2) to complete the entire gene sequence. The fixed sequence variations revealed by Pruett et al. (2001) occur towards the 3' end. Consequently, I designed primers to be situated downstream from Pruett et al.'s region of variability. In addition to capturing the four fixed base pair differences, these primers amplified to the end of cytochrome *b* in the event that this region would uncover additional variation. Forward primers for both the ND2 and ND6 regions were derived from previously published papers (Gibbs et al. 1996; Johnson et al. 1998) and were also used unmodified. However, I designed specific primers for the reverse compliment for each of these primers (CND2R, CND6C-L). Primers were designed to anneal to the target areas and amplify the complete gene sequence and to include base pairs flanking either side. When possible, they were also designed to anneal at higher temperatures, allowing for increased specificity.

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Figure 5. Novel gene order of Cuculidae, Falconiformes, Subocine Passeriformes, and Picidae (right) as compared to the typical gene order exhibited by most avian species (left). In novel gene order species, the Control Region has translocated in the 3' – 5' direction, and is positioned between $tRNA^{Thr}$ and $tRNA^{Pro}$. Thus, the control region is flanked by cytochrome *b* and ND6 in the 5'-3' direction, rather than being positioned between ND6 and 12s. (after Kvist 2000).

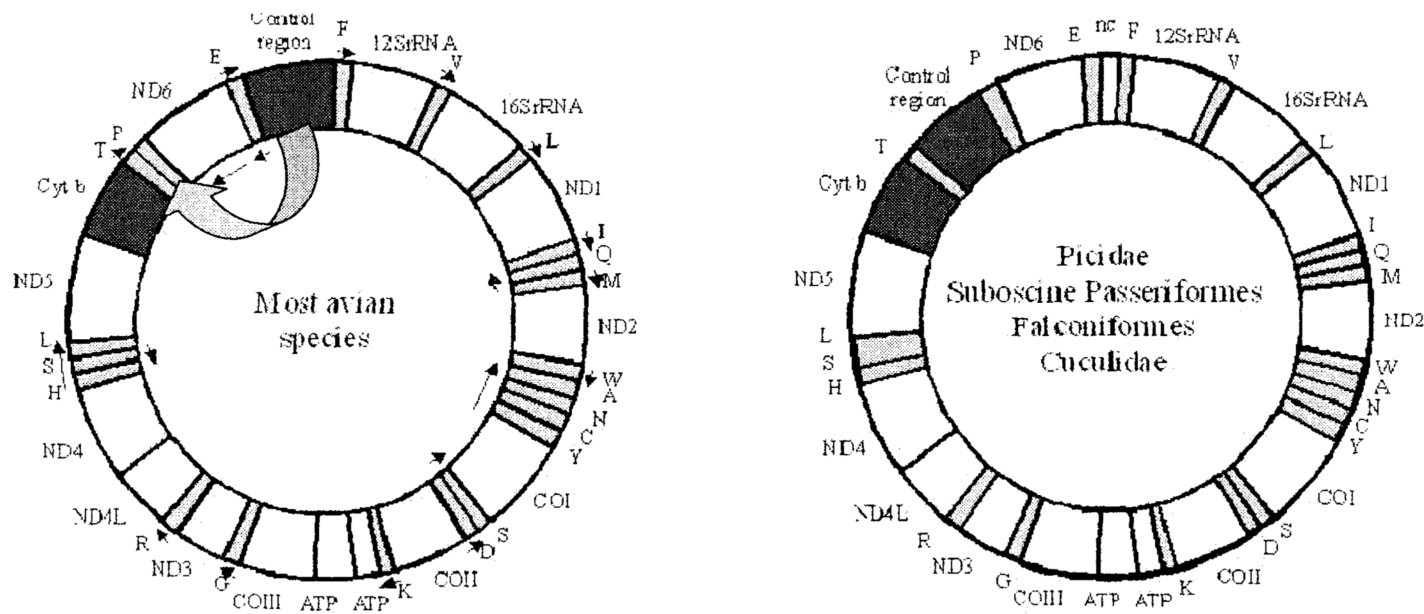

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Table 2. Complete list of primers used in this study. Previously published primers, in addition to several specifically designed primers, were used in this study to amplify all three target regions within the mtDNA genome of the Yellow-billed Cuckoo (*Coccyzus americanus*). Two internal primers (CYTB1 and CYTB2) were designed within cytochrome *b* due to primer specificity concerns and large amplicon size, which was found to be problematic.

Name	Target Region	Sequence (5′ – 3′)	Source
L14490	Cytochrome b	AACATCTCCGCATGATGAAA	Sorenson et al. (1999)
H16065	Cytochrome b	GGAGTCTTCAGTCTCTGGTTTACAAGAC	Sheldon et al. (2005)
CYTB1	Cytochrome b	CAAGGACCTAGTAGGATTCACT	This study
CYTB2	Cytochrome b	CACAAATCATCAATTCATCAA	This study
L5219	ND2	CCCATACCCCGAAAATGATG	Johnson et al. (1998)
CND2R	ND2	TGTAGGTTAGTATCCTGCGGGC	This study
ND6C-L	ND6	CGAGACAACCCACGGACAAG	Gibbs et al. (1996)
CND6R	ND6	TGTGTTGGGTGGGTTGGCGG	This study

PCR Protocol Development

Polymerase chain reaction (PCR) protocols were developed and refined for all three target regions through a series of trial amplifications conducted on five eastern Yellow-billed Cuckoo tissue samples. PCR amplification and all pre-sequencing PCR procedures were performed in the DNA laboratory located on the Lakehead University campus. DNA was sequenced in Paleo-DNA Laboratory Lakehead University. Initial amplifications were conducted using standard Platinum Taq conditions, whereby 3 μl of sample was added to 5 μl buffer, 1.3 μl DNTP, 1 μl primer, 2 μl MgCl, and 0.2 μl Taq, with the remaining volume containing 29.8 μl of H_20. PCR temperature profiles were performed on cycle 55mod45, which affected denaturation at 94°C for 120 seconds followed by 45 cycles of denaturation at 94°C for 60 seconds, annealing at 50-55°C for 60 seconds and extension at 72°C for 138 seconds. Products were subjected to electrophoresis through 0.375 g agarose gels in 49 mM Tris-acetate (pH 7.5) in the presence of 2 μl ethidium bromide. Following optimization of PCR protocols, the conditions that produced the sharpest and brightest amplifications were repeated for all remaining samples. Cycle sequencing was conducted using an ABI 3100 automated sequencer.

Data Analysis

Concatenated sequences 2,021 bp in length comprising portions of mitochondrial genes cytochrome *b* (949 bp), ND6 (560 bp), and ND2 (512 bp) were obtained for each sample by amplifying via the polymerase chain reaction using the primer pairs listed in Table 2. Sequences were individually aligned visually and assembled for

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all target regions using the software program Se-Al v. 2.0a11 (Rambaut 2002) with Yellow-billed Cuckoo sequences acquired from GenBank used as reference (Table 3). ND2 and ND6 sequences revealed no variation between eastern and western populations; hence, these data will not to be discussed further.

Two data sets comprising cytochrome *b* sequences were used in analysis — one set which consisted of only my data, and a second set that incorporated Pruett et al. (2001)'s sequences. The data were then subjected to the Maximum Likelihood (ML) method of phylogenetic reconstruction using PAUP*4.0b (Swofford 2002), in which the two separate analyses were conducted. Prior to the ML analyses, ModelTest 3.7 (Posada 2005) was used to determine the appropriate model of nucleotide substitution under the Akike Information Criterion, and to estimate the gamma distribution parameter of rate heterogeneity and proportion of invariant sites. The general-time-reversible (GTR) model of nucleotide substitution (Yang 1994), incorporating proportion of invariant sites (I) and four gamma distribution (G) values was used in both data sets. My data alone required the GTR+G model; my data in addition to Pruett et al. (2001) used GTR+I model. The ML analyses employed heuristic searches based on tree bisection and reconnection (TBR) branch swapping and 10 random stepwise additions of taxa. Tree searches were exhaustive. Trees were rooted using the Pearly-breasted Cuckoo (*Coccyzus julieni*; ANSP 4661), the sister species to the Yellow-billed Cuckoo (Hughes 2007) as outgroup. Uncorrected percent sequence divergences were determined for both cytochrome *b* data sets using PAUP*4.0b (Swofford 2002).

The data were subsequently translated into amino acid sequences using

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Table 3. Other sequences used in this study for alignment and/or data analysis. Pruett et al. (2001) samples that were included in the analysis are indicated by an asterik (*). All remaining *Coccyzus americanus and C. erythropthalmus* samples listed were used for alignment purposes only. Outgroup samples (*Coccyzus melacoryphus and C. juleni*) were used in both the analysis and for alignment of the data.

Taxon	Accession/Collection Number	Source	Region	Locality
Coccyzus melacoryphus	JMH20036	JMH	Cytochrome b	Paraguay
Coccyzus melacoryphus	JMH5958	JMH	Cytochrome b	Equador
Coccyzus julieni	ANSP4661	JMH	Cytochrome b	Equador
Coccyzus americanus	AY509696	Genbank	Cytochrome b	Wisconsin
Coccyzus americanus	AF204993	Genbank	Cytochrome b	—
Coccyzus americanus	AF082047	Genbank	Cytochrome b	—
Coccyzus americanus	MKP881	ROM	Cytochrome b	Maryland
Coccyzus americanus	AF249270*	Genbank	Cytochrome b	Minnesota
Coccyzus americanus	AF249271*	Genbank	Cytochrome b	Minnesota
Coccyzus americanus	AY046910*	Genbank	Cytochrome b	Vermont
Coccyzus americanus	AF249269*	Genbank	Cytochrome b	Alaska
Coccyzus americanus	AF249268*	Genbank	Cytochrome b	Alaska
Coccyzus americanus	AY046907*	Genbank	Cytochrome b	New Mexico
Coccyzus americanus	AY046906*	Genbank	Cytochrome b	New Mexico
Coccyzus americanus	AY046905*	Genbank	Cytochrome b	New Mexico

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Table 3 continued.

Taxon	Accession/Collection Number	Source	Region	Locality
Coccyzus americanus	AY046909*	Genbank	Cytochrome b	Mexico
Coccyzus americanus	AY046908*	Genbank	Cytochrome b	Mexico
Coccyzus erythropthalmus	AY274062	Genbank	ND2	Michigan

Specimen numbers refer to source collections: AF/AY, Genbank; MKP, Royal Ontario Museum; JMH, Janice Hughes, Lakehead University; ANSP, Academy of Natural Sciences, Philadelphia.

MacClade v. 4.03 (Maddison and Maddison 2000), then exported to PAUP*4.0b where third position transitions were excluded from both data sets. The third position-excluded data were then subjected to a second ModelTest and PAUP*4.0b ML analysis.

Relationships among haplotypes were inferred by constructing statistical parsimony networks using TCS v.1.18 (Clement et al. 2000) on all data sets — both my data and my data in addition to Pruett et al., and for the regular sequence data and sequence data with the third position excluded — resulting in four haplotype trees being generated. Individual haplotype base pair changes and haplotype groups were recorded for all four analyses.

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Results

Cytochrome b

Aligned sequences for my data had a base composition of 28.8% A, 35.0% C, 12.6% G, and 23.6% T. In the data set consisting of my data in addition to that of Pruett et al. (2001), sequences has a base composition of 27.7% A, 35.9% C, 13.0% G and 23.4% T. No insertions or deletions, frameshift mutations, or unexpected stop codons were found. Uncorrected pairwise "p" distance matrices for both data sets are indicated in Tables 4 and 5.

Aligned sequences revealed no consistent fixed base pair differences (bp) at the four variable sites identified by Pruett et al. (2001). The first of their fixed bp differences — a second position C to G change — was located in a region on the gene approximately 25 bp long that was found to be difficult to sequence reliably, causing recurring compressions in multiple taxa regardless of sequencing direction. This has been reported previously in other studies where avian cytochrome *b* was sequenced (Lanyon and Omland 1999). Pruett et al. (2001) may have had the same problem. In light of the fact that their purported fixed bp change at this position also causes a change in amino acid coding in a gene involved in cellular respiration — an unlikely and rare occurrence (Bjorkland 1999) — casts some doubt on Pruett et al.'s interpretation, thereby, making it unreliable. As a result, this particular site has been omitted from discussion due to the possibility of inconsistency in the gene sequence and potential reading errors (Lanyon and Omland 1999). Aside from this first purported fixed base pair change, which appeared to be generated randomly in

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Table 4. Uncorrected pairwise "p" distance matrix for cytochrome *b* sequences between all Yellow-billed Cuckoo (*Coccyzus americanus*) samples used in this study corresponding to maximum likelihood (ML) spanning tree (Figure 6). Pearly-breasted Cuckoo (*Coccyzus julieni*) is the outgroup.

	1	2	3	4	5	6	7
1 JMH C. julieni	-						
2 PAC381 California	0.01054	-					
3 7040 California	0.00843	0.00211	-				
4 207555 California	0.00738	0.00316	0.00105	-			
5 207553 California	0.00843	0.00421	0.00211	0.00105	-		
6 207596 Arizona	0.00843	0.00421	0.00211	0.00105	0.00211	-	
7 13712 Arizona	0.00843	0.00421	0.00211	0.00105	0.00211	0.00000	-
8 13715 Arizona	0.00738	0.00316	0.00105	0.00000	0.00105	0.00105	0.00105
9 207535 Arizona	0.00738	0.00316	0.00105	0.00000	0.00105	0.00105	0.00105
10 13719 Arizona	0.00738	0.00316	0.00105	0.00000	0.00105	0.00105	0.00105
11 20742 Arizona	0.01054	0.00632	0.00421	0.00316	0.00421	0.00421	0.00421
12 207594 Arizona	0.00843	0.00211	0.00000	0.00105	0.00211	0.00211	0.00211
13 207576 Arizona	0.01054	0.00632	0.00421	0.00316	0.00421	0.00421	0.00421
14 NK116168 New Mex	0.00843	0.00421	0.00211	0.00105	0.00211	0.00211	0.00211
15 NK11992 New Mex	0.00948	0.00527	0.00316	0.00211	0.00316	0.00316	0.00316
16 NK103366 New Mex	0.00948	0.00527	0.00316	0.00211	0.00316	0.00316	0.00316
17 NK 11964 Texas	0.00948	0.00527	0.00316	0.00211	0.00316	0.00316	0.00316
18 B21785 Texas	0.00843	0.00421	0.00211	0.00105	0.00211	0.00211	0.00211
19 B23431 Texas	0.00948	0.00527	0.00316	0.00211	0.00316	0.00316	0.00316
20 B37016 Texas	0.00843	0.00421	0.00211	0.00105	0.00211	0.00211	0.00211
21 EVL615 Louisiana	0.00948	0.00527	0.00316	0.00211	0.00316	0.00316	0.00316
22 UF44087 Florida	0.00738	0.00316	0.00105	0.00000	0.00105	0.00105	0.00105
23 UF44090 Florida	0.00738	0.00316	0.00105	0.00000	0.00105	0.00105	0.00105
24 2510 North Carolina	0.00738	0.00316	0.00105	0.00000	0.00105	0.00105	0.0105
25 619 New York	0.00738	0.00316	0.00105	0.00000	0.00105	0.00105	0.00105
26 JMH881 Maryland	0.00948	0.00527	0.00316	0.00211	0.00316	0.00316	0.00316

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Uncorrected "p" distance matrix (continued).

27 68159 Massachus	0.00738	0.00316	0.00105	0.00000	0.00105	0.00105	0.00105
28 441576 Wisconsin	0.00843	0.00421	0.00211	0.00105	0.00211	0.00211	0.00211
29 363714 Illinois	0.00948	0.00527	0.00316	0.00211	0.00316	0.00316	0.00316
30 429371 Iowa	0.00843	0.00421	0.00211	0.00105	0.00211	0.00211	0.00211
31 KU2690 Kansas	0.00738	0.00316	0.00105	0.00000	0.00105	0.00105	0.00105
32 KU27 Kansas	0.00738	0.00316	0.00105	0.00000	0.00105	0.00105	0.00105
33 Zo1.3.1 Alberta	0.01159	0.00738	0.00527	0.00421	0.00527	0.00527	0.00527

	8	9	10	11	12	13	14
8 13715 Arizona	-						
9 207535 Arizona	0.00000	-					
10 13719 Arizona	0.00000	0.00000	-				
11 20742 Arizona	0.00316	0.00316	0.00316	-			
12 207594 Arizona	0.00105	0.00105	0.00105	0.00421	-		
13 207576 Arizona	0.00316	0.00316	0.00316	0.00632	0.00421	-	
14 NK116168 New Mex	0.00105	0.00105	0.00105	0.00211	0.00211	0.00421	-
15 NK11992 New Mex	0.00211	0.00211	0.00211	0.00316	0.00316	0.00527	0.00105
16 NK103366 New Mex	0.00211	0.00211	0.00211	0.00527	0.00316	0.00527	0.00316
17 NK11964 Texas	0.00211	0.00211	0.00211	0.00316	0.00316	0.00527	0.00105
18 B21785 Texas	0.00105	0.00105	0.00105	0.00421	0.00211	0.00421	0.00211
19 B23431 Texas	0.00211	0.00211	0.00211	0.00316	0.00316	0.00527	0.00105
20 B37016 Texas	0.00105	0.00105	0.00105	0.00421	0.00211	0.00421	0.00211
21 EVL615 Louisiana	0.00211	0.00211	0.00211	0.00527	0.00316	0.00527	0.00316
22 UF44087 Florida	0.00000	0.00000	0.00000	0.00316	0.00105	0.00316	0.00105
23 UF44090 Florida	0.00000	0.00000	0.00000	0.00316	0.00105	0.00316	0.00105
24 2510 North Carolina	0.00000	0.00000	0.00000	0.00316	0.00105	0.00316	0.00105
25 619 New York	0.00000	0.00000	0.00000	0.00316	0.00105	0.00316	0.00105
26 JMH881 Maryland	0.00211	0.00211	0.00211	0.00527	0.00316	0.00527	0.00316
27 68159 Massachus	0.00000	0.00000	0.00000	0.00316	0.00105	0.00316	0.00105
28 441576 Wisconsin	0.00105	0.00105	0.00105	0.00421	0.00211	0.00421	0.00211

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Uncorrected "p" distance matrix (continued).

29 363714 Illinois	0.00211	0.00211	0.00211	0.00316	0.00316	0.00527	0.00105
30 429371 Iowa	0.00105	0.00105	0.00105	0.00421	0.00211	0.00421	0.00211
31 KU2690 Kansas	0.00000	0.00000	0.00000	0.00316	0.00105	0.00316	0.00105
32 KU27 Kansas	0.00000	0.00000	0.00000	0.00316	0.00105	0.00316	0.00105
33 Z01.3.1 Alberta	0.00421	0.00421	0.00421	0.00738	0.00527	0.00738	0.00527

	15	16	17	18	19	20	21
15 NK11992 New Mex	-						
16 NK103366 New Mex	0.00421	-					
17 NK11964 Texas	0.00211	0.00421	-				
18 B21785 Texas	0.00316	0.00316	0.00316	-			
19 B23431 Texas	0.00211	0.00421	0.00211	0.00105	-		
20 B37016 Texas	0.00316	0.00316	0.00316	0.00211	0.00316	-	
21 EVL615 Louisiana	0.00421	0.00421	0.00211	0.00316	0.00421	0.00316	-
22 UF44087 Florida	0.00211	0.00211	0.00211	0.00105	0.00211	0.00105	0.00211
23 UF44090 Florida	0.00211	0.00211	0.00211	0.00105	0.00211	0.00105	0.00211
24 2510 North Carolina	0.00211	0.00211	0.00211	0.00105	0.00211	0.00105	0.00211
25 619 New York	0.00211	0.00211	0.00211	0.00105	0.00211	0.00105	0.00211
26 JMH881 Maryland	0.00421	0.00421	0.00421	0.00316	0.00421	0.00316	0.00421
27 68159 Massachus	0.00211	0.00211	0.00211	0.00105	0.00211	0.00105	0.00211
28 441576 Wisconsin	0.00316	0.00316	0.00316	0.00211	0.00316	0.00211	0.00316
29 363714 Illinois	0.00211	0.00421	0.00211	0.00316	0.00211	0.00316	0.00421
30 426371 Iowa	0.00316	0.00316	0.00105	0.00211	0.00316	0.00211	0.00105
31 KU2690 Kansas	0.00211	0.00211	0.00211	0.00105	0.00211	0.00105	0.00211
32 KU27 Kansas	0.00211	0.00211	0.00211	0.00105	0.00211	0.00105	0.00211
33 Z01.3.1 Alberta	0.00632	0.00632	0.00632	0.00527	0.00632	0.00316	0.00632

	22	23	24	25	26	27	28
22 UF44087 Florida	-						
23 UF44090 Florida	0.00000	-					

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Uncorrected "p" distance matrix (continued).

24 2510 North Carolina	0.00000	0.00000	-				
25 619 New York	0.00000	0.00000	0.00000	-			
26 JMH881 Maryland	0.00211	0.00211	0.00211	0.00211	-		
27 68159 Massachus	0.00000	0.00000	0.00000	0.00000	0.00211	-	
28 441576 Wisconsin	0.00105	0.00105	0.00105	0.00105	0.00316	0.00105	-
29 363714 Ilinois	*0.00211*	*0.00211*	*0.00211*	*0.00211*	*0.00421*	*0.00211*	*0.00316*
30 429371 Iowa	0.00105	0.00105	0.00105	0.00105	0.00316	0.00105	0.00211
31 KU2690 Kansas	0.00000	0.00000	0.00000	0.00000	0.00211	0.00000	0.00105
32 KU27 Kansas	0.00000	0.00000	0.00000	0.00000	0.00211	0.00000	0.00105
33 Z01.3.1 Alberta	0.00421	0.00421	0.00421	0.00421	0.00632	0.00421	0.00527

	29	30	31	32	33
29 363714 Illinois	-				
30 429371 Iowa	0.00316	-			
31 KU2690 Kansas	0.00211	0.00105	-		
32 KU27 Kansas	0.00211	0.00105	0.00000	-	
33 Z01.3.1 Alberta	0.00632	0.00527	0.00421	0.00421	-

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Table 5. Uncorrected pairwise "p" distance matrix for cytochrome *b* sequences between all Yellow-billed Cuckoo (*Coccyzus americanus*) samples used in this study corresponding to maximum likelihood (ML) spanning tree (Figure 7). Pruett et al. (2001) data is incorporated and identified by samples with the GenBank accessions AF and AY. Pearly-breasted Cuckoo (*Coccyzus julieni*) is the outgroup.

	1	2	3	4	5	6	7
1 JMH4661 C. julieni	-						
2 AF249269 Alaska	0.00904	-					
3 AF249268 Alaska	0.01017	0.00113	-				
4 AF249271 Minnesota	0.01695	0.00791	0.00904	-			
5 AF249270 Minnesota	0.01469	0.00565	0.00678	0.00226	-		
6 AY046910 Vermont	0.01356	0.00452	0.00565	0.00339	0.00113	-	
7 AY046909 Mexico	0.01130	0.00452	0.00565	0.01243	0.01017	0.00904	-
8 AY046908 Mexico	0.01017	0.00339	0.00452	0.01130	0.00904	0.00791	0.00113
9 AY046907 New Mex	0.01130	0.00226	0.00113	0.01017	0.00791	0.00678	0.00678
10 AY046906 New Mex	0.01017	0.00113	0.00226	0.00904	0.00678	0.00565	0.00565
11 AY046905 New Mex	0.00904	0.00000	0.00113	0.00791	0.00565	0.00452	0.00452
12 PAC381 California	0.01130	0.00226	0.00339	0.01017	0.00791	0.00678	0.00678
13 7040 California	0.00904	0.00000	0.00113	0.00791	0.00565	0.00452	0.00452
14 207555 California	0.00791	0.00113	0.00226	0.00904	0.00678	0.00565	0.00339
15 207553 California	0.00904	0.00226	0.00339	0.01017	0.00791	0.00678	0.00452
16 207596 Arizona	0.00904	0.00226	0.00339	0.01017	0.00791	0.00678	0.00452
17 13712 Arizona	0.00904	0.00226	0.00339	0.01017	0.00791	0.00678	0.00452
18 13715 Arizona	0.00791	0.00113	0.00226	0.00904	0.00678	0.00565	0.00339
19 207535 Arizona	0.00791	0.00113	0.00226	0.00904	0.00678	0.00565	0.00339
20 13719 Arizona	0.00791	0.00113	0.00226	0.00904	0.00678	0.00565	0.00339
21 20742 Arizona	0.01130	0.00452	0.00565	0.00565	0.00339	0.00226	0.00678
22 207594 Arizona	0.00904	0.00000	0.00113	0.00791	0.00565	0.00452	0.00452
23 207576 Arizona	0.00904	0.00226	0.00339	0.1017	0.00791	0.00678	0.00452
24 NK116168 New Mex	0.00904	0.00226	0.00339	0.00791	0.00565	0.00452	0.00452

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Uncorrected “p” distance matrix (continued).

25 NK11992 New Mex	0.01017	0.00339	0.00452	0.00904	0.00678	0.00565	0.00565
26 NK103366 New Mex	0.01017	0.00339	0.00452	0.01130	0.00904	0.00791	0.00565
27 NK11964 Texas	0.01017	0.00339	0.0452	0.00904	0.00678	0.00565	0.00565
28 B21785 Texas	0.00904	0.00226	0.00339	0.01017	0.00791	0.00678	0.00452
29 B23431 Texas	0.01017	0.00339	0.00452	0.00904	0.00678	0.00565	0.00565
30 B37016 Texas	0.00791	0.00113	0.00226	0.00904	0.00678	0.00565	0.00339
31 EVL615 Louisiana	0.01017	0.00339	0.00452	0.01130	0.00904	0.00791	0.00565
32 UF44087 Florida	0.00791	0.00113	0.00226	0.00904	0.00678	0.00565	0.00339
33 UF44090 Florida	0.00791	0.00113	0.00226	0.00904	0.00678	0.00565	0.00339
34 2510 North Carolina	0.00791	0.00113	0.00226	0.00904	0.00678	0.00565	0.00339
35 619 New York	0.00791	0.00113	0.00226	0.00904	0.00678	0.00565	0.00339
36 JMH881 Maryland	0.01017	0.00339	0.00452	0.01130	0.00904	0.00791	0.00565
37 68159 Massachu	0.00791	0.00113	0.00226	0.00904	0.00678	0.00565	0.00339
38 441576 Wisconsin	0.00904	0.00226	0.00339	0.01017	0.00791	0.00678	0.00452
39 363714 Illinois	0.01017	0.00339	0.00226	0.00904	0.00678	0.00565	0.00565
40 429371 Iowa	0.00904	0.00226	0.00339	0.01017	0.007901	0.00678	0.00452
41 KU2690 Kansas	0.00791	0.00113	0.00226	0.00904	0.00678	0.00565	0.00339
42 KU27 Kansas	0.00791	0.01130	0.00226	0.00904	0.00678	0.00565	0.00339
43 Z01.3.1 Alberta	0.01017	0.00339	0.00452	0.01130	0.00904	0.00791	0.00565

	8	9	10	11	12	13	14
8 AY046908 Mexico	-						
9 AY046907 Mexico	0.00565	-					
10 AY046906 New Mex	0.00452	0.00339	-				
11 AY046905 New Mex	0.00339	0.00226	0.00113	-			
12 PAC381 California	0.00565	0.00452	0.00339	0.00226	-		
13 7040 California	0.00339	0.00226	0.00113	0.00000	0.00226	-	
14 207555 California	0.00226	0.00339	0.00226	0.00113	0.00339	0.00113	-
15 207553 California	0.00339	0.00452	0.00339	0.00226	0.00452	0.00226	0.00113
16 207596 Arizona	0.00339	0.00226	0.00339	0.00226	0.00452	0.00226	0.00113

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Uncorrected "p" distance matrix (continued).

17 13712 Arizona	0.00339	0.00226	0.00339	0.00226	0.00452	0.00226	0.00113
18 13715 Arizona	0.00226	0.00339	0.00226	0.00113	0.00339	0.00113	0.00000
19 207535 Arizona	0.00226	0.00339	0.00226	0.00113	0.00339	0.00113	0.00000
20 13719 Arizona	0.00226	0.00339	0.00226	0.00113	0.00339	0.00113	0.00000
21 20742 Arizona	0.00565	0.00678	0.00565	0.00452	0.00678	0.00452	0.00339
22 207594 Arizona	0.00339	0.00226	0.00113	0.00000	0.00226	0.00000	0.00113
23 207576 Arizona	0.00339	0.00452	0.00339	0.00226	0.00452	0.00226	0.00113
24 NK116168 New Mex	0.00339	0.00452	0.00339	0.00226	0.00452	0.00226	0.00113
25 NK11992 New Mex	0.00452	0.00565	0.00452	0.00339	0.00565	0.00339	0.00226
26 NK103366 New Mex	0.00452	0.00565	0.00452	0.00339	0.00565	0.00339	0.00226
27 NK11964 Texas	0.00452	0.00565	0.00452	0.00339	0.00565	0.00339	0.00226
28 B21785 Texas	0.00339	0.00452	0.00339	0.00226	0.00452	0.00226	0.00113
29 B23431 Texas	0.00452	0.00565	0.00452	0.00339	0.00565	0.00339	0.00226
30 B37016 Texas	0.00226	0.00339	0.00226	0.00113	0.00339	0.00113	0.00000
31 EVL615 Louisiana	0.00452	0.00565	0.00452	0.00339	0.00565	0.00339	0.00226
32 UF44087 Florida	0.00226	0.00339	0.00226	0.00113	0.00339	0.00113	0.00000
33 UF44090 Florida	0.00226	0.00339	0.00226	0.00113	0.00339	0.00113	0.00000
34 2510 North Carolina	0.00226	0.00339	0.00226	0.00113	0.00339	0.00113	0.00000
35 619 New York	0.00226	0.00339	0.00226	0.00113	0.00339	0.00113	0.00000
36 JMH881 Maryland	0.00452	0.00565	0.00452	0.00339	0.00565	0.00339	0.00226
37 68159 Massachu	0.00226	0.00339	0.00226	0.00113	0.00339	0.00113	0.00000
38 441576 Wisconsin	0.00339	0.00452	0.00339	0.00226	0.00452	0.00226	0.00113
39 363714 Illinois	0.00452	0.00339	0.00452	0.00339	0.00565	0.00339	0.00226
40 429371 Iowa	0.00339	0.00452	0.00339	0.00226	0.00452	0.00226	0.00113
41 KU2690 Kansas	0.00226	0.00339	0.00226	0.00113	0.00339	0.00113	0.00000
42 KU27 Kansas	0.00226	0.00339	0.00226	0.00113	0.00339	0.00113	0.00000
43 Z.01.3.1 Alberta	0.00452	0.00565	0.00452	0.00339	0.00565	0.00339	0.00226

	15	16	17	18	19	20	21
15 207553 California	-						

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Uncorrected “p” distance matrix (continued).

	22	23	24	25	26	27	28
16 207576 Arizona	0.00226	-					
17 13712 Arizona	0.00226	0.00000	-				
18 13715 Arizona	0.00113	0.00113	0.00113	-			
19 207535 Arizona	0.00113	0.00113	0.00113	0.00000	-		
20 13719 Arizona	0.00113	0.00113	0.00113	0.00000	0.00000	-	
21 20742 Arizona	0.00452	0.00452	0.00452	0.00339	0.00339	0.00339	-
22 207594 Arizona	0.00226	0.00226	0.00226	0.00113	0.00113	0.00113	0.00452
23 207576 Arizona	0.00226	0.00226	0.00226	0.00113	0.00113	0.00113	0.00452
24 Nk116168 New Mex	0.00226	0.00226	0.00226	0.00113	0.00113	0.00113	0.00226
25 NK11992 New Mex	0.00339	0.00339	0.00339	0.00226	0.00226	0.00226	0.00339
26 NK103366 New Mex	0.00339	0.00339	0.00339	0.00226	0.00226	0.00226	0.00565
27 NK11964 Texas	0.00339	0.00339	0.00339	0.00226	0.00226	0.00226	0.00339
28 B21785 Texas	0.00226	0.00226	0.00226	0.00113	0.00113	0.00113	0.00452
29 B23431 Texas	0.00339	0.00339	0.00339	0.00226	0.00226	0.00226	0.00339
30 B37016 Texas	0.00113	0.00113	0.00113	0.00000	0.00000	0.00000	0.00339
31 EVL615 Louisiana	0.00339	0.00339	0.00339	0.00226	0.00226	0.00226	0.00565
32 UF44087 Florida	0.00113	0.00113	0.00113	0.00000	0.00000	0.00000	0.00339
33 UF44090 Florida	0.00113	0.00113	0.00113	0.00000	0.00000	0.00000	0.00339
34 2510 North Carolina	0.00113	0.00113	0.00113	0.00000	0.00000	0.00000	0.00339
35 619 New York	0.00113	0.00113	0.00113	0.00000	0.00000	0.00000	0.00339
36 JMH881 Maryland	0.00339	0.00339	0.00339	0.00226	0.00226	0.00226	0.00565
37 68159 Massachu	0.00113	0.00113	0.00113	0.00000	0.00000	0.00000	0.00339
38 441576 Wisconsin	0.00226	0.00226	0.00226	0.00113	0.00113	0.00113	0.00452
39 363714 Illinois	0.00339	0.00339	0.00339	0.00226	0.00226	0.00226	0.00339
40 429371 Iowa	0.00226	0.00226	0.00226	0.00113	0.00113	0.00113	0.00452
41 KU2690 Kansas	0.00113	0.00113	0.00113	0.0000	0.0000	0.00000	0.00339
42 KU27 Kansas	0.00113	0.00113	0.00113	0.0000	0.0000	0.0000	0.00339
43 Z.01.3.1 Alberta	0.00339	0.00339	0.00339	0.00226	0.00226	0.00226	0.00565

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Uncorrected "p" distance matrix (continued).

22 207594 Arizona	-						
23 207576 Arizona	0.00226	-					
24 NK116168 New Mex	0.00226	0.00226	-				
25 NK11992 New Mex	0.00339	0.00339	0.00113	-			
26 NK103366 New Mex	0.00339	0.00339	0.00339	0.00452	-		
27 NK11992 Texas	0.00339	0.00339	0.00113	0.00226	0.00452	-	
28 B21785 Texas	0.00226	0.00226	0.00226	0.00339	0.00339	0.00339	-
29 B23431 Texas	0.00339	0.00339	0.00113	0.00226	0.0452	0.00226	0.00113
30 B37016 Texas	0.00113	0.00113	0.00113	0.00226	0.00226	0.00226	0.00113
31 EVL615 Louisiana	0.00339	0.00339	0.00339	0.00452	0.00452	0.00226	0.00339
32 UF44087 Florida	0.00113	0.00113	0.00113	0.00226	0.00226	0.00226	0.00113
33 UF44090 Florida	0.00113	0.00113	0.00113	0.00226	0.00226	0.00226	0.00113
34 2510 North Carolina	0.00113	0.00113	0.00113	0.00226	0.00226	0.00226	0.00113
35 619 New York	0.00113	0.00113	0.00113	0.00226	0.00226	0.00226	0.00113
36 JMH881 Maryland	0.00339	0.00339	0.00339	0.00452	0.00452	0.00452	0.00339
37 68159 Masschu	0.00113	0.00113	0.00113	0.00226	0.00226	0.00226	0.00113
38 441576 Wisconsin	0.00226	0.00226	0.00226	0.00339	0.00339	0.00339	0.00226
39 363714 Illinois	0.00339	0.00339	0.00113	0.00226	0.00452	0.00226	0.00339
40 429371 Iowa	0.00226	0.00226	0.00226	0.00339	0.00339	0.00113	0.00226
31 KU2690 Kansas	0.00113	0.00113	0.00113	0.00226	0.00226	0.00226	0.00113
32 KU27 Kansas	0.00113	0.00113	0.00113	0.00226	0.00226	0.00226	0.00113
32 Z01.3.1 Alberta	0.00339	0.00339	0.00339	0.00452	0.00452	0.00452	0.00339

	29	30	31	32	33	34	35
29 B23431 Texas	-						
30 B37016 Texas	0.00226	-					
31 EVL615 Louisiana	0.00452	0.00226	-				
32 UF44087 Florida	0.00226	0.00000	0.00226	-			
33 UF44090 Florida	0.00226	0.00000	0.00226	0.00000	-		
34 2510 North Carolina	0.00226	0.00000	0.00226	0.00000	0.00000	-	

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Uncorrected "p" distance matrix (continued).

35 619 New York	0.00226	0.00000	0.00226	0.00000	0.00000	0.00000	-
36 JMH881 Maryland	0.00452	0.00226	0.00452	0.00226	0.00226	0.00226	0.00226
37 68159 Massachu	0.00226	0.00000	0.00226	0.00000	0.00000	0.00000	0.00000
38 441576 Wisconsin	0.00339	0.00113	0.00339	0.00113	0.00113	0.00113	0.00113
39 363714 Illinois	0.00226	0.00226	0.00452	0.00226	0.00226	0.00226	0.00226
40 429371 Iowa	0.00339	0.00113	0.00113	0.00113	0.00113	0.00113	0.00113
41 KU2690 Kansas	0.00226	0.00000	0.00226	0.00000	0.00000	0.00000	0.00000
42 KU27 Kansas	0.00226	0.00000	0.00226	0.00000	0.00000	0.00000	0.00000
43 Z01.3.1 Alberta	0.00452	0.00226	0.00452	0.00226	0.00226	0.00226	0.00226

	36	37	38	39	40	41	42
36 JMH881 Maryland	-						
37 68159 Massachu	0.00226	-					
38 441576 Wisconsin	0.00339	0.00113	-				
39 363714 Illinois	0.00452	0.00226	0.00339	-			
40 429371 Iowa	0.00339	0.00113	0.00226	0.00339	-		
41 KU2690 Kansas	0.00226	0.00000	0.00113	0.00226	0.00113	-	
42 KU27 Kansas	0.00226	0.00000	0.00113	0.00226	0.00113	0.00000	-
43 Z01.3.1 Alberta	0.00452	0.00226	0.00339	0.00452	0.00339	0.00226	0.00226

	43
43 Z01.3.1 Alberta	-

some of the samples, all eastern and western samples contained none of the differences identified by Pruett et al. (2001) to separate the two subspecies (or haplotypes) of Yellow-billed Cuckoo (*C. a. americanus* and *C. a. occidentalis*). Furthermore, the four base pair difference was also only found in their eastern samples, a result I was not able to replicate. In contrast, all samples identified by Pruett et al. (2001) as belonging to the western haplotype more closely matched my data.

All phylogenetic trees generated on whole sequence data in both data sets — one consisting of only my data and a second set that also incorporated Pruett et al. sequences (Figures 6 and 7) — in addition to trees generated with a second analyses with third codon positions excluded from both data sets (Figures 8 and 9) showed no substantial divergence among eastern and western Yellow-billed Cuckoos. In my cytochrome *b* data set, ML trees (see Figure 6) and TCS haplotype trees generated on these data sets (see Figure 10 and Table 6) revealed a number of samples grouped together according to region but, primarily, the data showed no east–west divergence. Within the ML tree (Figure 6), one large clade and five smaller clades were apparent. The "western" designated samples made up the majority of the resolved clades, where individuals from Texas; Arizona; Arizona and California; Arizona, New Mexico, and Texas; and Alberta and Texas were grouped. Two Texas individuals formed an internal clade within this larger clade. The only exception to these western groupings was one eastern-designated individual (Illinois 363714), which was positioned in the largest clade formed with individuals from Arizona, New Mexico, and Texas. This is likely an artifact of the limited amount of

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Figure 6. Maximum likelihood tree of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples used in this study, based on a 949 base pair cytochrome *b* sequence. Tree rooted with Pearly-breasted Cuckoo (*Coccyzus julieni*) as outgroup. See Appendix 1 for further explanation on location and accession of samples.

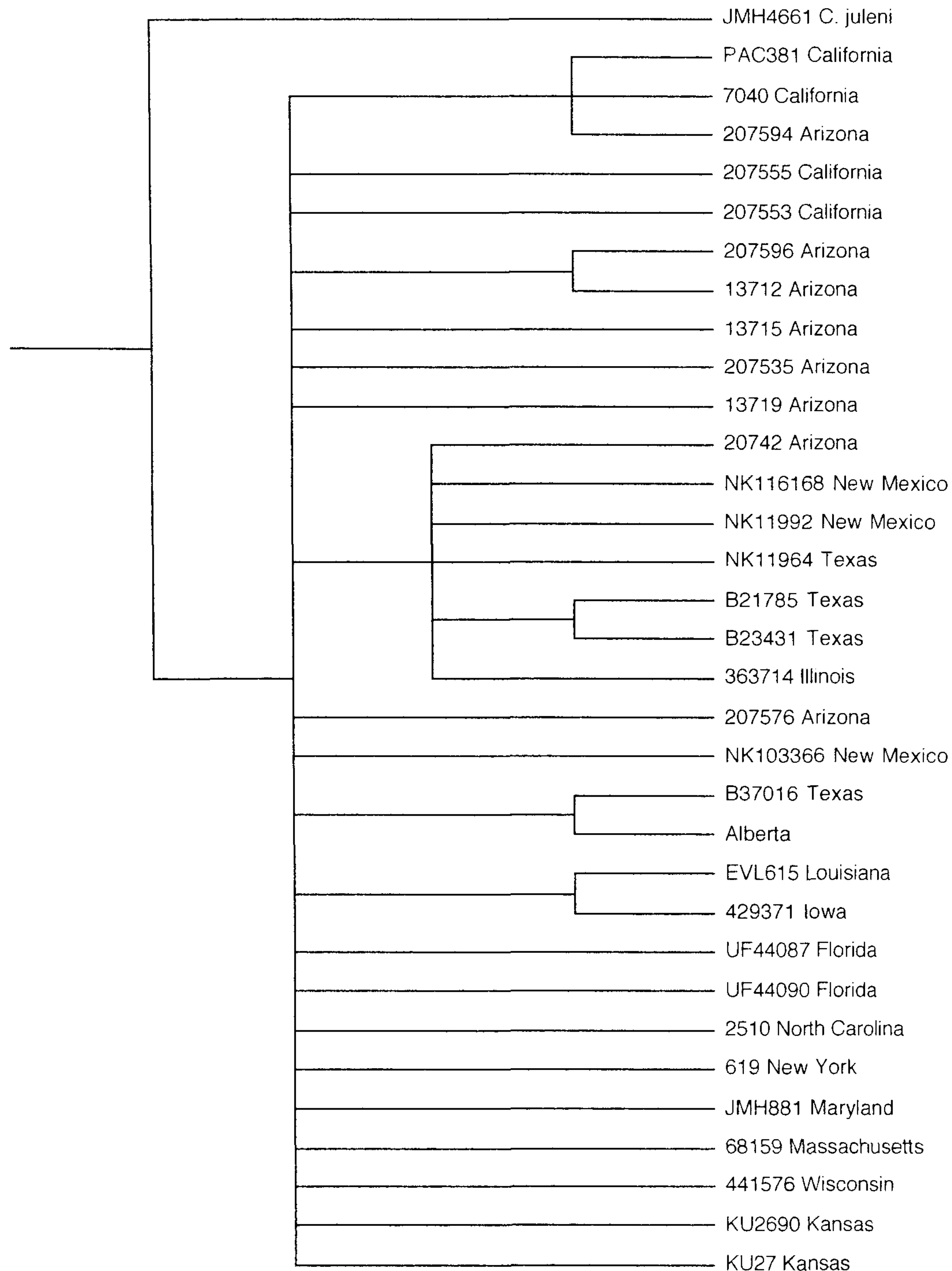

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Figure 7. Maximum likelihood tree of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples used in this study, based on a 885 base pair sequence of cytochrome *b*. Pruett et al. (2001) data is incorporated and identified by samples with the GenBank accessions AF and AY. Tree rooted with Pearly-breasted Cuckoo (*Coccyzus julieni*) as outgroup. See Appendix 1 for further explanation on location and accession of samples.

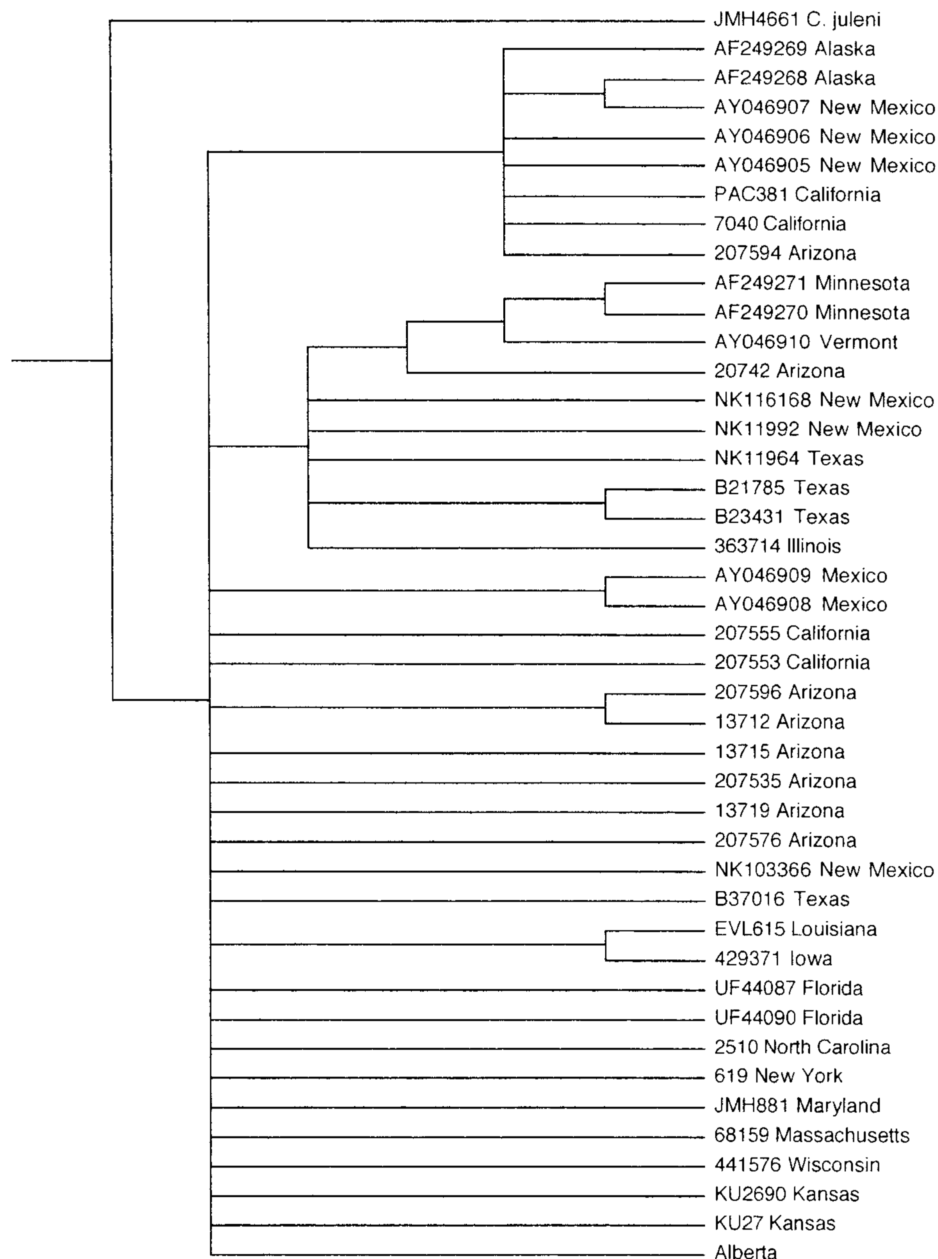

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Figure 8. Maximum likelihood tree of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples used in this study, based on a 949 base pair sequence of cytochrome *b* with third codon positions excluded. Tree rooted with Pearly-breasted Cuckoo (*Coccyzus julieni*) as outgroup. See Appendix 1 for further explanation on location and accession of samples.

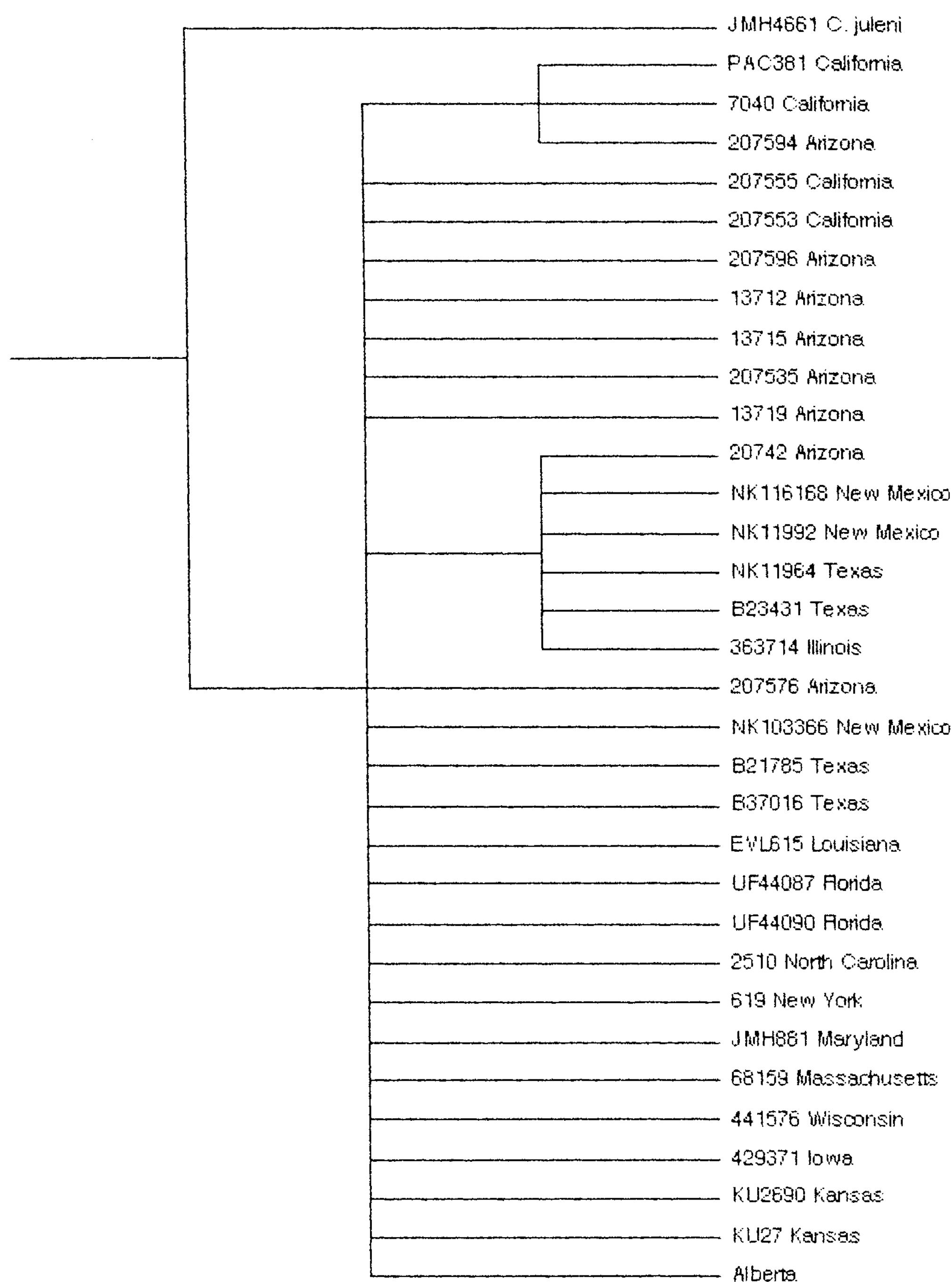

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Figure 9. Maximum likelihood tree of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples used in this study, based on a 885 base pair sequence of cytochrome *b* with third codon positions excluded. Pruett et al. (2001) data is incorporated and identified by samples with the GenBank accessions AF and AY. Tree rooted with Pearly-breasted Cuckoo (*Coccyzus julieni*) as outgroup. See Appendix 1 for further explanation on location and accession of samples.

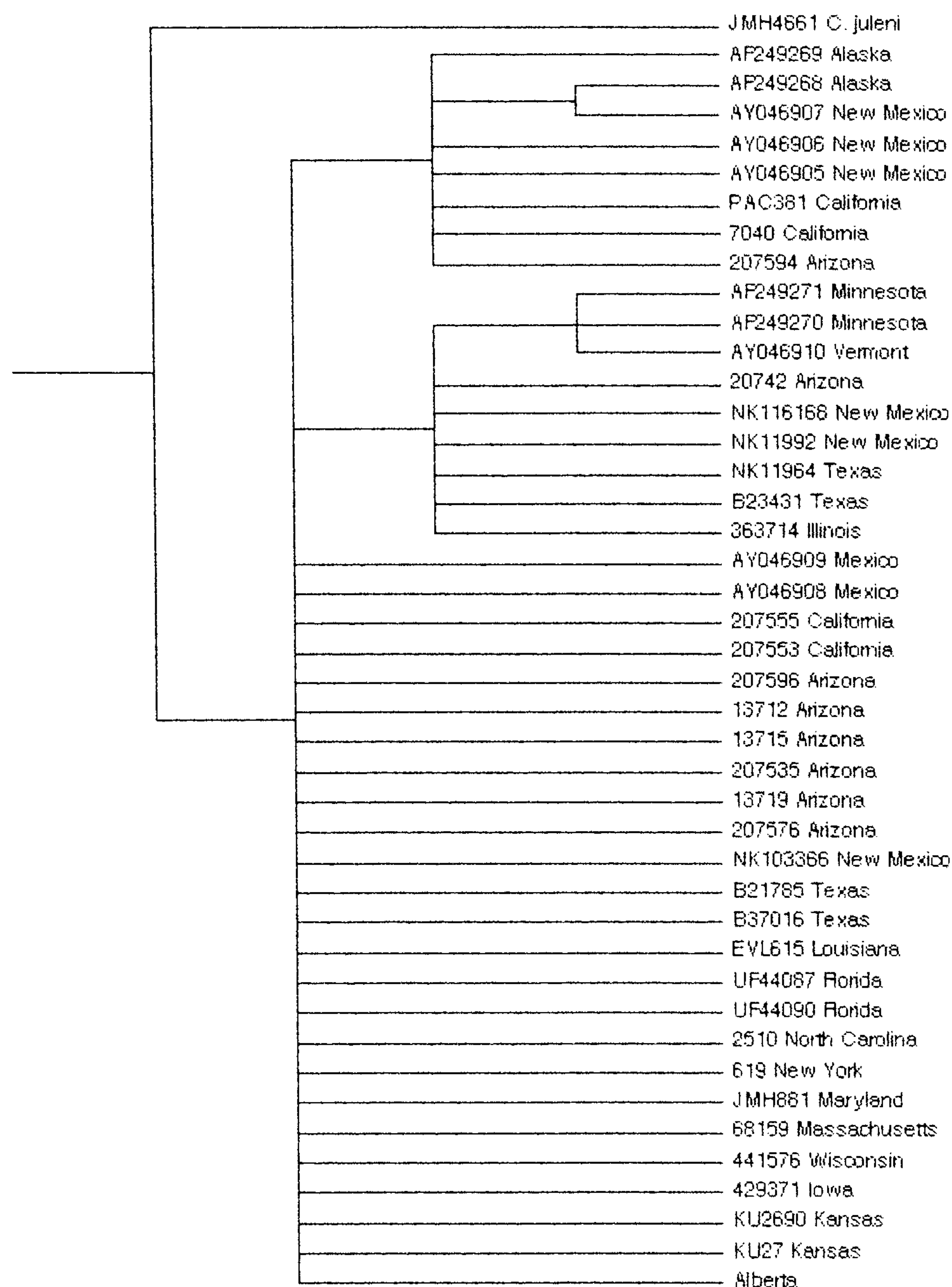

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Figure 10. Statistical parsimony network haplotype diagram of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples used in this study excluding Pruett et al. (2001) data, based on a 949 base pair sequence of cytochrome *b*. See Table 6 for an explanation of numbered sample clusters and haplotype designations.

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Table 6. Haplotype designations of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples used in this study corresponding to statistical parsimony network haplotype diagram (see Figure 10), based on a 949 base pair sequence of cytochrome *b*.

Haplotype	Accession	Locality	Haplotype	Accession	Locality
1	Z01.3.1	Alberta	10	UF44090	Florida
2	JMH881	Maryland		2510	North Carolina
3	EVL615	Louisiana		619	New York
4	NK103366	New Mexico		68159	Massachusetts
5	207576	Arizona		KU2690	Kansas
6	20742	Arizona		KU27	Kansas
7	207596	Arizona	11	207553	California
	13712	Arizona	12	NK116168	New Mexico
8	PAC381	California	13	B-21785	Texas
9	7040	California	14	B-37016	Texas
	207594	Arizona	15	441576	Wisconsin
10	207555	California	16	429371	Iowa
	13715	Arizona	17	NK11992	New Mexico
	207535	Arizona	18	NK11964	Texas
	13719	Arizona	19	363714	Illinois
	UF44087	Florida	20	B-23431	Texas

Specimen numbers refer to source collections: UAM, University of Alaska Museum, Fairbanks; AMNH, American Museum of Natural History, New York; NK, University of New Mexico, Albuquerque; FMNH, Field Museum of Natural History, Chicago; MBR, University of Kansas Natural History Museum, Lawrence; UF, Florida Museum of Natural History, Gainesville; UWBM, University of Washington Burke Museum of Natural History, Seattle; ANSP, Academy of Natural Sciences of Philadelphia; RAM, Royal Alberta Museum, Edmonton; LSUMZ, Louisiana State University Museum of Natural History, Baton Rouge; MH, Murrelet Halterman, University of Nevada, Reno.

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variation inherent in my data set; in this case, one shared base pair difference, particularly a first or third position transition, among samples could cause the phylogenetic software to cluster these haplotypes artificially. Further analysis using other genes would be required to resolve this alleged relationship. Finally, one small clade on the ML tree was made up of two eastern-designated individuals from Louisiana and Iowa.

When third codon positions were excluded from the data, the resulting ML tree (Figure 8) lost much of the structure resolved in these smaller clades. Only two clades were formed in this second ML tree: one comprising one Arizona and two California individuals, and a larger clade with one Arizona, two New Mexican, two Texas, and one Illinois sample. Two Texas samples that had grouped together previously no longer formed a clade when third positions were excluded.

The haplotype tree generated in TCS on full sequence data (Figure 10; Table 6) revealed 20 haplotypes among the data but, again, no significant east–west division among samples. The largest haplotype grouping (haplotype 10) consisted of 11 individuals of which there were both eastern- and western-designated samples possessing that haplotype. The remaining haplotypes consisted of single individuals, except haplotypes 7 and 9, which were attributed to two samples each. When third positions were excluded from the data, seven haplotypes were generated among the data (Figure 11; Table 7). The largest haplotype grouping (haplotype 4) represented 21 samples of both eastern and western distribution. The remaining haplotypes grouped samples according to region or individual basis, where western individuals in Arizona, New Mexico, and Texas shared haplotype 5

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Figure 11. Statistical parsimony network haplotype diagram of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples used in this study excluding Pruett et al. (2001) data, based on a 949 base pair sequence of cytochrome *b*. Third codon positions were excluded from the data. See Table 7 for an explanation of numbered sample clusters and haplotype designations.

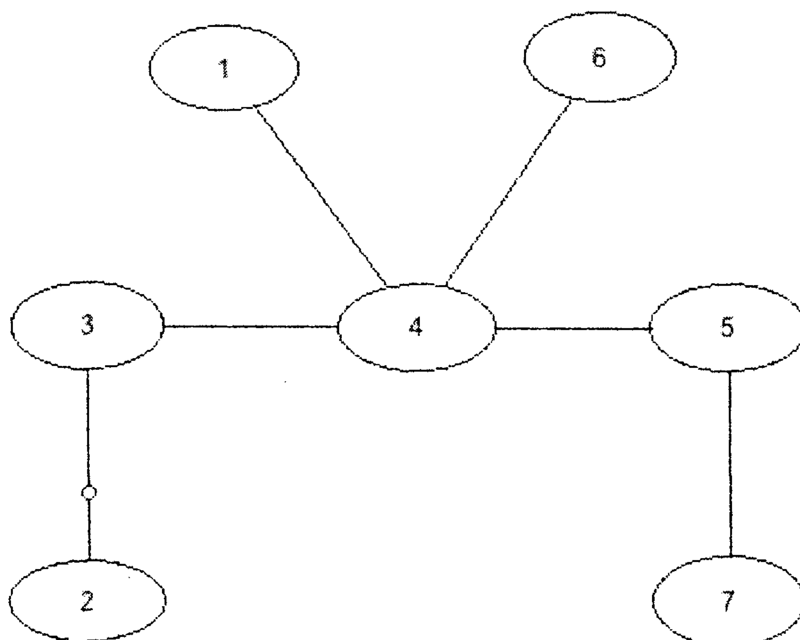

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Table 7. Haplotype designations of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples used in this study corresponding to statistical parsimony network haplotype diagram (see Figure 11), based on a 949 base pair sequence of cytochrome b. Third codon positions were excluded from the data.

Haplotype	Accession	Locality	Haplotype	Accession	Locality
1	207576	Arizona	4	UF44090	Florida
2	PAC381	California		2510	North Carolina
3	7040	California		619	New York
	207594	Arizona		JMH881	Maryland
4	207555	California		68159	Massachusetts
	207553	California		441576	Wisconsin
	207596	Arizona		429371	Iowa
	13712	Arizona		KU2690	Kansas
	13715	Arizona		KU27	Kansas
	207535	Arizona	5	20742	Arizona
	13719	Arizona		NK116168	New Mexico
	NK103366	New Mexico		NK11992	New Mexico
	B-21785	Texas		NK11964	Texas
	B-37016	Texas		B-23431	Texas
	EVL615	Louisiana	6	Z01.3.1	Alberta
	UF44087	Florida	7	363714	Illinois

Specimen numbers refer to source collections: UAM, University of Alaska Museum, Fairbanks; AMNH, American Museum of Natural History, New York; NK, University of New Mexico, Albuquerque; FMNH, Field Museum of Natural History, Chicago; MBR, University of Kansas Natural History Museum, Lawrence; UF, Florida Museum of Natural History, Gainesville; UWBM, University of Washington Burke Museum of Natural History, Seattle; ANSP, Academy of Natural Sciences of Philadelphia; POMA, Provincial Museum of Alberta, Edmonton; LSUMZ, Louisiana State University Museum of Natural History, Baton Rouge; MH, Murrelet Halterman, University of Nevada, Reno.

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and in California and Arizona shared haplotype 3. In the entire data set (including Pruett et al. sequences), ML trees (Figure 7) and TCS haplotype trees (see Figure 12 and Table 8) grouped Pruett et al.'s eastern samples together, thereby, separating them from my samples. When the third codon positions were excluded from the data, ML trees (Figure 9) and haplotype trees (Figure 13; Table 9) revealed that these eastern sequences lost some variability, but still maintained their deeper structure and formed haplotypes that were divergent from those apparent in my data.

The ML tree generated from all cytochrome *b* sequences (Figure 7) features two large clades and eight smaller clades. Pruett et al. "eastern" samples were incorporated within the largest clade, which also included individuals from Arizona, New Mexico, Texas, and Illinois from my data set. Furthermore, these eastern individuals formed an internal clade within this larger clade that was, otherwise, divergent from my data. Within this eastern clade, the Vermont sample is sister to paired Minnesota samples. An Arizona individual is sister to this grouping. Within the larger clade, there is also a small clade comprising two Texas individuals. Alaska and New Mexico samples from Pruett et al. cluster with my California and Arizona samples to form the second largest clade in a internal clade is formed from one Alaska and one New Mexico individual. The remaining small clades in the ML tree include Pruett et al.'s two Mexican samples, two of my Arizona individuals and, finally, two eastern individuals from Louisiana and Iowa.

When third positions were excluded from the data, the resulting ML tree (Figure 9) lost all of its structure generated by smaller clades present in the data set.

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Figure 12. Statistical parsimony network haplotype diagram of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples based on a 885 base pair sequence of cytochrome *b*. Pruett et al. (2001) data is incorporated, where circles denote haplotypes identified within their data. See Table 8 for an explanation of numbered sample clusters and haplotypes.

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Table 8. Haplotype designations of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples used in this study corresponding to statistical parsimony network haplotype diagram (see Figure 12), based on a 885 base pair sequence of cytochrome *b*. Pruett et al. (2001) data set is incorporated and identified by asterisk (*) and samples with the GenBank accessions AF and AY.

Haplotype	Accession	Locality	Haplotype	Accession	Locality
1	AY046909*	Mexico	17	207535	Arizona
2	AY046908*	Mexico		13719	Arizona
3	Z01.3.1	Alberta		B37016	Texas
4	JMH881	Maryland		UF44087	Florida
5	EVL615	Louisiana		UF44090	Florida
6	NK103366	New Mexico		2510	North Carolina
7	20742	Arizona		619	New York
8	AF249271*	Minnesota		68159	Massachusetts
9	AF249270*	Minnesota		KU2690	Kansas
10	AY046910*	Vermont		KU27	Kansas
11	NK116168	New Mexico		13715	Arizona
12	207596	Arizona	18	AY046907*	New Mexico
	13712	Arizona	19	207553	California
13	PAC381	California	20	207576	Arizona
14	AF249269*	Alaska	21	B21785	Texas
	AY046905*	New Mexico	22	441576	Wisconsin
	7040	California	23	429371	Iowa
	207594	Arizona	24	NK11992	New Mexico
15	AF249268*	Alaska	25	NK11964	Texas
16	AY046906*	New Mexico	26	363714	Illinois
17	207555	California	27	B23431	Texas

Specimen numbers refer to source collections: UAM, University of Alaska Museum, Fairbanks; AMNH, American Museum of Natural History, New York; NK, University of New

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Mexico, Albuquerque; FMNH, Field Museum of Natural History, Chicago; MBR, University of Kansas Natural History Museum, Lawrence; UF, Florida Museum of Natural History, Gainesville; UWBM, University of Washington Burke Museum of Natural History, Seattle; ANSP, Academy of Natural Sciences of Philadelphia; RAM, Royal Alberta Museum, Edmonton; LSUMZ, Louisiana State University Museum of Natural History, Baton Rouge; MH, Murrelet Halterman, University of Nevada, Reno.

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Figure 13. Statistical parsimony network haplotype diagram of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples, based on an 885 base pair sequence of cytochrome *b* with third codon positions excluded. Pruett et al. (2001) data is incorporated, where circles denote haplotypes identified within their data. See Table 9 for an explanation of numbered sample clusters and haplotype designations.

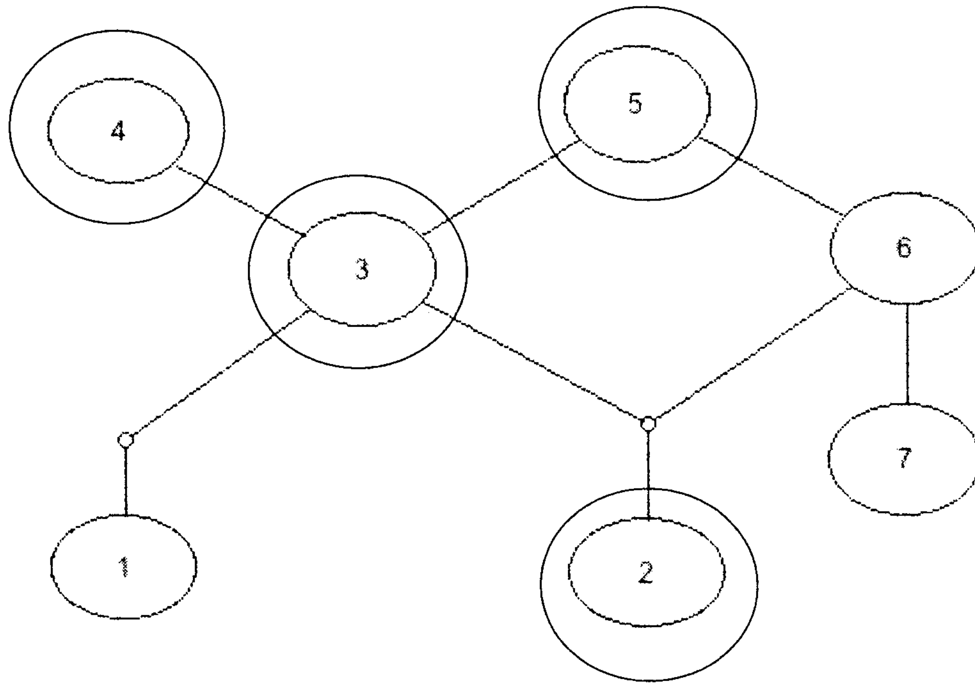

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Table 9. Haplotype designations of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples used in this study corresponding to statistical parsimony network haplotype diagram (see Figure 13), based on a 885 base pair sequence of cytochrome *b*. Third codon positions were excluded from the data. Pruett et al. (2001) data set is incorporated and identified by asterisk (*) and samples with the GenBank accessions AF and AY.

Haplotype	Accession	Location	Haplotype	Accession	Location
1	PAC381	California	5	NK103366	New Mexico
2	AF249271*	Minnesota		B21785	Texas
	AF249270*	Minnesota		B37016	Texas
	AY046910*	Vermont		EVL615	Louisiana
3	AF249269*	Alaska		UF44087	Florida
	AY046906*	New Mexico		UF44090	Florida
	AY046905*	New Mexico		2510	North Carolina
	7040	California		619	New York
	207594	Arizona		JMH881	Maryland
4	AF249268*	Alaska		68159	Massachusetts
	AY046907*	New Mexico		441576	Wisconsin
5	AY46909*	Mexico		429371	Iowa
	AY046908*	Mexico		KU2690	Kansas
	207555	California		KU27	Kansas
	207553	California		Z01.3.1	Alberta
	207596	Arizona	6	20742	Arizona
	13712	Arizona		NK116168	New Mexico
	13715	Arizona		NK11992	New Mexico
	207535	Arizona		NK11964	Texas
	13719	Arizona		B23431	Texas
	207576	Arizona	7	363714	Illinois

Specimen numbers refer to source collections: UAM, University of Alaska Museum, Fairbanks; AMNH, American Museum of Natural History, New York; NK, University of New

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Mexico, Albuquerque; FMNH, Field Museum of Natural History, Chicago; MBR, University of Kansas Natural History Museum, Lawrence; UF, Florida Museum of Natural History, Gainesville; UWBM, University of Washington Burke Museum of Natural History, Seattle; ANSP, Academy of Natural Sciences of Philadelphia; RAM, Royal Alberta Museum, Edmonton; LSUMZ, Louisiana State University Museum of Natural History, Baton Rouge; MH, Murrelet Halterman, University of Nevada, Reno.

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The two large clades (those incorporating Pruett et al. data) remained but with less variation, whereas, all the structure among my data disappeared. The largest clade included an internal clade comprising Pruett et al. eastern individuals; however, the relationship between these three samples was now unresolved. The second largest clade (incorporating Pruett et al.'s Alaska and New Mexican samples) retained its structure despite the exclusion of third positions. However, the only resolved internal clade was formed from one Alaskan and one Pruett New Mexican sample from Pruett et al. (2001).

Twenty-seven haplotypes were found within the complete cytochrome *b* data set (including Pruett et al. sequences; Figure 12; Table 8). The largest haplotype grouping (haplotype 17) included individuals from my specimens attributed to both the eastern and western regions. Pruett et al.'s eastern individuals comprised different haplotypes for each sample (haplotypes 8, 9, and 10). In addition, the Mexican samples were haplotypes 1 and 2, and New Mexican samples were haplotypes 14, 16 and 18. Their Alaska samples were split: one was haplotype 15, the other shared haplotype 14 with New Mexico, California and Arizona samples. My data represented the remaining haplotype groupings with samples from both the east and west represented by their own unique haplotype.

With third positions excluded, the haplotype diagram (Figure 13; Table 9) designated 7 haplotypes among the complete data set. Pruett et al.'s eastern samples shared their own haplotype (haplotype 2) apart from the rest of my data. One of Pruett et al.'s Alaska and two of their New Mexico individuals shared haplotype 3 with my California and Arizona samples. These Alaska and New

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Mexican samples from Pruett et al. also had their own haplotype (haplotype 4), while their Mexican samples clustered with the largest haplotype grouping (haplotype 5), which was formed from the majority of my samples. The remaining haplotype groupings in the diagram comprised individuals from California, Arizona, New Mexico, Texas, and Illinois.

For all haplotype trees generated on whole sequence and with third codon positions excluded, individual haplotype base pair changes for my data are illustrated in Tables 10 and 11; and my data in addition to Pruett et al. in Tables 12 and 13, where their four fixed base pair differences are identified by asterisk.

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Table 10. Individual haplotype base pair changes for statistical parsimony network haplotype diagram (see Figure 10) of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples based on a 949 base pair sequence of cytochrome *b*.

Position	Haplotype bp change	Position	Haplotype bp change
158	C – T	632	A – G
194	G – A	644	C – A
194	G – A	644	C – A
254	G – A	683	G – C
260	G – A	684	C – T
261	T – G	706	G – C
284	C – T	782	A – C
348	G – C	787	G – A
350	T – C	818	C – A
493	G – C	836	C – A
493	C – G	878	A – G
493	C – G	915	G – T
518	G – A	917	G – A
566	T -C	947	A – G
620	T – C		

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Table 11. Individual haplotype base pair changes for statistical parsimony network haplotype diagram (see Figure 11) of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples based on a 949 base pair sequence of cytochrome *b*, where all third codon positions were excluded from the data.

Position	Haplotype base pair change
174	T – G
232	G – C
329	C – G
456	C – T
471	G – C
525	A – C
610	T – G

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Table 12. Individual haplotype base pair changes for statistical parsimony network haplotype diagram (see Figure 12) of all Yellow-billed Cuckoo (*Coccyzus americanus*) samples based on a 885 base pair sequence of cytochrome *b*. Pruett et al. (2001) data set is incorporated, where the four fixed haplotype bp differences examined by Pruett et al. (2001) are indicated by an asterisk (*).

Position	Haplotype bp change	Position	Haplotype bp change
161	C – T	575	A – G
197	G – A	623	T – C
197	G – A	623	T – C
257	G – A	635	A – G
263	G – A	645*	G – A*
264	T – G	647	C – A
287	C – T	647	C – A
299	A – C	686	C – G
311	A – G	687	C – T
351	G – C	687	C – T
353	T – C	709	G – C
395	A – T	709	G – C
496	C – G	785	A – C
496*	C – G*	821	C – A
496	G – C	839	C – A
509	C – T	845*	A – C*
521	G – A	881	A – G
569*	T – C*	884	A – G

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Table 13. Individual haplotype base pair changes for statistical parsimony network haplotype diagram (see Figure 13) of all Yellow-billed Cuckoo samples based on a 885 base pair sequence of cytochrome *b*, where all third codon positions were excluded from the data. Four fixed haplotype bp differences examined by Pruett et al. (2001) are indicated by an asterisk (*).

Position	Haplotype base pair change
176	T – G
234	G – C
331*	C – G*
331	C – G
430*	G – A*
458	C – T
458	C – T
473	C – G
473	G – C

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Discussion

Although currently listed as Status 1 (Critically Imperiled, Endangered) in California and Threatened in Arizona, there is still no measure of protection for the Yellow-billed Cuckoo in New Mexico, Texas, Utah, and Colorado (Hughes 1999). Furthermore, the western Yellow-billed Cuckoo has been under review for federal protection since 1986. In the ensuing time since petitioning for listing began, however, the western populations have plummeted by over 95 percent as rivers have been dammed and diverted, and streamside forests have been grazed by livestock or removed for recreational and urban development. The cuckoos have also been severely impacted by pesticide spraying of sphinx moth caterpillars (*Eumorpha fasciata*), their primary food source. (Suckling 2001).

As a result, there are now less than 50 pairs remaining in California and even fewer in Nevada, Utah, Idaho, Montana, Wyoming, and Colorado (Suckling 2001). The largest isolated population occurs in Arizona, where 168 pairs and 80 single birds were located in a 1999 state-wide survey that covered 427 kilometers of river and creek bottoms (USFWS 2001). The only other substantial populations — about 200 pairs each — are found in western portions of New Mexico and Texas (Suckling 2001). Based on current estimates, there are no more than about 1,000 western Yellow-billed Cuckoos.

In 2001, Pruett et al. used DNA sequence data to document substantial divergence between eastern and western populations that supported recognition of *C. a. occidentalis* as a Distinct Vertebrate Population Segment. However, no new

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petition to list the western Yellow-billed Cuckoo under the ESA has been submitted since 1998. Each year, the western Yellow-billed Cuckoo is included in the USFWS Federal Register *Endangered and Threatened Wildlife and Plants; Review of Species That Are Candidates or Proposed for Listing as Endangered or Threatened* and in *Annual Description of Progress on Listing Actions*, however, it is simply mentioned with no recommended action towards the listing of the subspecies.

Role of Subspecies and Evolutionary Significant Units

The term Evolutionary Significant Unit (ESU) was first brought to the attention of a broad audience of ecologists and evolutionary biologists by Ryder (1986). The concept was developed to provide a rational basis for prioritizing taxa for conservation effort, and to ensure that evolutionary heritage is recognized and protected and that the evolutionary potential inherent across the set of ESUs is maintained (Moritz 1994). The ESU has been associated with Distinct Vertebrate Population Segment (DPS), as designated by the U.S. Fish and Wildlife Service (USFWS), which receives protection under the ESA. The term is also used in a variety of less formal contexts worldwide (Crandall et al. 2000).

The abundance of Yellow-billed Cuckoos throughout its range reveals a conflict between legal and biological concerns, because the ESA does not recognize the western population as endangered — according to current species' listings and the USFWS — if populations elsewhere in the range are healthy. Not until the western populations of Yellow-billed Cuckoo are given subspecific status can it be

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thoroughly evaluated for its merit as an ESU and DPS and then, finally, given protection under the ESA.

Recent controversy concerns whether or not currently recognized subspecies can be considered equivalent to ESUs. Zink (2004) suggested that this may not be the case. Furthermore, he stated that repeatedly designating taxonomic units as subspecies primarily for conservation ultimately results in misleading conservation policy, an example being the threatened California Gnatcatcher (*Polioptila californica*; Zink et al. 2000; Zink 2004). In this study, mtDNA control region, t-RNA^{Glu}, ND2, ND3, and ND6 gene sequences from 64 individuals taken throughout the species' range, and their concomitant geographical pattern of haplotype diversity, implied that Californian Coastal Sage Scrub Gnatcatchers were not a genetically distinct population but, rather, resulted from population growth and recent range expansion northward from Southern Baja California into southwestern California. Thus, northern populations are not a unique biodiversity component. It is true that many historically defined subspecies, such as the California Gnatcatcher, may need to be reevaluated when conservation plans are an issue; however, Zink (2004) fails to acknowledge that the subspecies designation could allow a threatened taxon to receive recognition that would initiate conservation efforts.

A case in point is the Cape Sable Seaside Sparrow (*Ammodramus maritimus mirabilis*). Nelson et al. (2000) identified a distinct matrilineal clade within 'Atlantic' Cape Sable Sparrows using mtDNA sequences that was highly divergent from Gulf Coast seaside sparrows. Now currently listed as Endangered on USFWS Threatened and Endangered Species System (TESS), the Cape Sable Sparrow is

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awaiting Federal ESA legislation. The North American Marbled Murrelet (*Brachyramphus marmoratus marmoratus*) has also received conservation incentives through the recognition of traditional subspecies designation. The species was first listed as Threatened in California, Oregon, and Washington and Threatened on the Committee on the Status of Endangered Wildlife in Canada in 1992. Since that time, there has been an explosion of studies on the species, particularly regarding its obligate use of old growth forests habitat for nesting (Agler et al. 1998, Burger et al. 2000; Jodice and Collopy 2000; Rodray et al. 2003). Consequently, the murrelet has become a flagship species in efforts to prevent the logging of old-growth forests along the Pacific Coast — an obvious source of controversy among opposing interests that include environmentalists, foresters, and urban developers (Ward 2002). Furthermore, Canadian environmentalist groups and researchers have been able to devote a considerable effort to Marbled Murrelet research and monitoring that has proven essential for evaluating the effectiveness of conservation efforts in maintaining this unique form.

Zink (2004) adds that among 21 temperate, continentally-distributed subspecies identified as threatened or endangered in North America only 13 have been evaluated for mtDNA variation; 12 others demonstrate a lack of divergence. Somewhat contradictory, however, are his statements that this conclusion cannot compare subspecies in general, because some studies of endangered taxa do not include the entire species' range. Such is the case for Pruett et al. (2001) who attempted to describe genetic variation within Yellow-billed Cuckoos using a sample size of only 10 individuals: three eastern and seven purported western individuals.

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Although the vicariant model of speciation — which involves gradual divergence of two moderate-to-large populations over extended periods of separation by extrinsic barriers to gene flow — is widely accepted for many vertebrate animals, it is not satisfactory for all taxa, particularly birds (Friesen and Anderson 1997). Birds encounter few obstacles to dispersal, yet their rates of molecular divergence are higher than many other vertebrate taxa (Friesen and Anderson 1997). For example, mtDNA sequence variation has delineated at least five subspecies of sandhill cranes (*Grus canadensis*), a mid-continent species (Glenn et al. 2000), three of which are currently listed under the ESA. It is obvious that some continental avian species can evolve considerable genetic diversity, despite the opinions of Zink (2004).

Speciation and Geographic Variation in Yellow-billed Cuckoos

Historically, subspecies were defined among birds by differences observed in colour and pattern of plumage and/or differences in size and proportion of various body regions that were believed to be genetically based. Intraspecific differences in other aspects of species' biology were also studied as they may coincide with physical differences implying substantial genetic divergence (Franzreb and Laymon 1993). For example, western Yellow-billed Cuckoos differ from eastern populations in plumage and osteological morphology, breeding chronology, location and chronology of migration, and visual and vocal social behaviours (Franzreb and Laymon 1993). The extent to which local populations differ genetically can determine their potential for local adaptation, evolution, and speciation, which is

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subsequently affected by variables such as gene flow, effective population size, and selection. Genetic divergence among populations that have not attained equilibrium between mutation, migration, and genetic drift, may also reflect historical factors, such as population bottlenecks and changes in distribution (Friesen et al. 1996). Hence, the myriad of morphological and behavioural differences observed between eastern and western Yellow-billed Cuckoos, provides strong support to the recognition of two genetically distinct taxonomic units, even though available molecular data has not yet revealed it.

Inconsistency Among the Cytochrome b Data Sets

Neither data set used in my study — my data alone and my data plus Pruett et al. sequences — revealed east-west divergence among Yellow-billed Cuckoos in maximum likelihood and statistical parsimony network analyses. Furthermore, my cytochrome *b* data set failed to find the four fixed base pair differences described by Pruett et al. that were used to delineate the two populations. Interestingly, haplotypes derived from Pruett et al.'s western samples (Alaska, New Mexico, and Mexico) strongly resembled the most prevalent haplotypes shared by eastern and western individuals from my data set alone. Only the three samples designated eastern in their study — two from Minnesota and one Vermont (Genbank accessions: AF249270, AF249271 and AY46910) — contained the alleged four fixed base pair differences. It is obvious that Pruett et al. (2001) identified the standard haplotype as eastern based solely on these three samples and assumed that the western haplotype was divergent. The errors in their conclusions were compounded

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by using the Black-billed Cuckoo as an outgroup to root their trees. Hughes (2007) demonstrated that this species is genetically divergent from the Yellow-billed Cuckoo and, therefore, does not represent the optimal outgroup species. My data set compiled from 31 samples representing a much wider geographic coverage clearly demonstrate that the only "distinct" haplotype belongs to Pruett et al.'s three eastern samples; their western individuals merely exhibit the standard haplotypes possessed by all my samples. Even my samples collected regionally close to those of Pruett et al. (Minnesota = Wisconsin; Vermont = Connecticut) fail to "match" their eastern cuckoos. This anomaly would have been apparent to Pruett et al. had they sequenced additional eastern samples granted from a greater range of sources.

Evidence suggests that the source of Pruett et al's specimens may provide an explanation for this mystery. It is apparent that the three eastern samples in question were all granted from the University of Alaska Museum (UAM); they have sequential accession numbers and were likely prepared concurrently. At the time of their preparation, UAM was using dimethyl sulfoxide (DMSO) — a buffer known to produce DNA damage under some circumstances — to preserve their samples. Hence, there is a strong possibility that the purported four fixed based pair changes represent damage in Pruett et al.'s data set.

Possibility of DMSO Damage to mtDNA

DNA in biological samples decays rapidly after death and ensuing damage is manifested in many forms (Gilbert et al. 2003a). Strand fragmentation can be caused by endonuclease activity or hydrolytic attacks that lead to depurination of

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adenine or guanine nucleotides, thus, weakening the DNA backbone leading to its destruction (Lindahl 1993). The majority of postmortem DNA damage, however, occurs as double-stranded breaks and oxidative base modifications, both of which can prevent subsequent enzymatic replication (Gilbert et al. 2003b). Much of the oxidative DNA modification, along with the presence of free radicals, damage the DNA by modifying cytosine and thymine nucleotides and sugar residues of the DNA backbone, all of which block the activity of enzymes of the polymerase chain reaction (Gilbert et al. 2003a). Free radicals are produced in living cells by normal metabolism and by exogenous sources, such as carcinogenic compounds and ionizing radiation. Reactive oxygen species, particularly the highly reactive hydroxyl radical (*OH), causes the most damage to DNA. When the (*OH) free radical is liberated in solution, it can attack many chemical bonds within DNA, including the A – T and C – G bonds of the DNA template, with the potential to cause irreversible damage (Dizdarouglu et al. 2002). Free radicals may persist after the organism's death generating further damage *postmortem* concomitant with the biological processes of necrosis and decay. In the genetic study of archived specimens or museum tissue samples, damage may manifest from *postmortem* handling or storage of the tissue in preservatives, buffers, or solutions where DNA fragmentation continues as a dynamic process, dependant upon the type of fixative, type of buffer, length of exposure to the fixative, and tissue type (Rose et al. 1995).

DMSO ($(CH_3)_2SO$) is a hydrogen-bonding oxidant that binds to DNA, thereby separating the double stranded structure into single strands. It is an aprotic solvent with a highly polar S=O group and two hydrophobic CH_3 groups, where its polar site

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has a strong affinity for water, forming strong hydrogen bonds, and its non-polar sites can cause effects of hydrophobic hydration and hydrophobic association of DMSO molecules (Vaisman and Berkowitz 1992). DMSO has been used extensively in the PCR procedure as an additive to help reduce the amount of secondary structure within the DNA template. It also acts as a free radical scavenger (Brayton 1986).

Several years ago, DMSO was a popular choice of preservative as a mixed dilution in the storage of museum tissue samples (D. Dittmann *pers comm.*). It has been found, however, that significant alterations in protein secondary structure are induced by DMSO and DMSO/water mixtures (Jackson and Mantsch 1991), and long-term storage of tissue samples in DMSO or aqueous DMSO solutions can cause oxidative damage. Oxidative damage can generate G – C and A – C transversions via a 5-hydroxyuracil and 2-hydroxyadenine intermediates, respectively. Similarly A – G and C – T transitions can occur via a 8-hydroxyadenine and 5-hydroxycytosine intermediates, respectively. Other forms of damage generating A – G and C – T transitions can proceed through hydrolytic intermediates hypoxanthine and uracil (Hansen et al. 2001; Dizdaroglu et al. 2002).

Records suggest that Pruett et al.'s eastern samples were preserved in an aqueous DMSO solution, upon which damage to the DNA template may have created two transversions and, possibly, two transitions comprising the four fixed base pair differences suggested to be haplotype changes. Furthermore, the eastern samples were derived from salvaged cuckoo carcasses donated to the University of Alaska Museum from the Bell Museum of Natural History. It is possible that severe

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decay and damage to the DNA template took place during the natural chemical processes associated with death, and any damage was further compounded by the storage medium (i.e., an aqueous DMSO solution) and/or storage conditions. Other specimens used by Pruett et al. were apparently frozen without prior preservation except those from the Museum of Southwestern Biology (AY46905, AY46906, AY46907), which were preserved in 100% ethanol (EtOH), according to the policy of that institution. No sequences derived from these specimens exhibited the fixed base pair changes. Furthermore, all specimens comprising my data set were shipped in 100% EtOH or HCl Tris lysis buffer solution typically used for avian tissue samples. Although, I received two tissue grants from the University of Alaska, these specimens did not prove to be problematic in this regard. One sample (UAM/CLD399) was prepared many years ago prior to DMSO being used at the UAM; this being evidenced by the low accession number attributed to the specimen. I sequenced successfully cytochrome *b* from this tissue sample, the resulting sequences conformed to my data, which did not show the fixed base pair changes. A second specimen granted from the University of Alaska (UAM6953) could not be amplified or sequenced due to some undetermined inhibitory factor. Based on the accession number, this inhibitory factor may have, indeed, been DMSO.

Pruett et al.'s two transversions, a second position C – G and a third position A – C, can clearly be attributed to damage caused by DMSO, based on the type of base pair change and known activity of DMSO in aqueous solutions on the DNA structure. The two transitions may also have been caused by a combination of chemical processes involving the aqueous DMSO solution. Additionally they may be

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attributable to reading errors on the DNA template caused by standard polymerase errors (Hansen et al. 2002), as well as free radical induced damage by a combination of conducive storage conditions such as the formation of ice crystals within tissues during cycles of freezing and thawing (Rose et al. 1995).

Further support for these conclusions is provided by the specific nature of the base pair changes; two purported changes in eastern samples — one transition and one transversion — imply translations that would result in coding for a different amino acids. Viable changes to amino acid sequences are extremely rare occurrences evolutionarily because most result in deleterious effects (Bjorkland 1999) and, thus, would be highly unlikely events in such closely-related forms. It is doubtful that any of Pruett's four fixed base pair differences have any phylogenetic significance, and the aforementioned arguments provide substantial reason to cast doubt on the integrity of their data set.

Third Position Substitutions in Cytochrome b

In many phylogenetic analysis using DNA sequences, third positions are often downweighted or excluded, giving higher weight to second and first positions. This is based on the belief that frequently-occurring third position substitutions may imply greater misleading information than more slowly-evolving first and second positions (Bjorklund 1999). In phylogenetic studies, "signal" from similarities due to shared ancestry must be distinguished from "noise" originating from similarities due to convergence. One way of improving the signal-to-noise ratio is to identify characters more likely to provide a strong signal and discard those that potentially demonstrate

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convergence (Bjorkland 1999). Edwards et al. (1991) examined both negative and positive effects for disregarding third positions in phylogenetic analysis. They determined that third positions can result in disinformation in deep branches that conflicts with structure supported by first and second positions. In contrast, they also concluded that third position transversions can provide phylogenetic information when comparing more closely-related sequences. However, phylogenetic analysis based solely on first and second positions suggested a biologically unreasonable grouping. The nucleotide composition in avian cytochrome *b* is highly skewed in third positions, and there is evidence for considerable codon bias. For example, the bias against guanines observed in third positions of avian DNA may suggest that these substitutions may be under considerable selection and, thus, may not be as neutral as previously thought (Bjorkland 1999). Within my data set, third position substitutions occurred at intervals, both in samples designated eastern and western. Due to the controversy surrounding their utility in resolving evolutionary relationships among closely-related taxa, however, I chose to perform two analyses on the data sets — one that included third positions, and one that excluded them from the data — in the hope that an optimal tree would be generated.

In all cases, the omission of third position variation weakened the phylogenetic structure inherent in the data set. Cytochrome *b* sequences within this species exhibited little variation at onset (see Tables 4 and 5), so reducing the number of variable positions available for tree reconstruction undoubtedly reduced resolution. However, support for a few robust clades persisted thus attesting to some supportable phylogenetic signal present in the data set.

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Haplotype Subgroups in the Western Region

Both maximum likelihood and statistical parsimony network reconstructions revealed some consistent associations among my western specimens, even when third positions were removed from the analyses. These are likely indicative of phylogenetic signal reflecting true evolutionary relationships among these individuals. For example, one specimen from Alaska (AY249268) and another from New Mexico (AY046907) maintained a sister relationship in which they shared a unique haplotype. Yellow-billed Cuckoos do not regularly occur as far north as Alaska; consequently, the individual collected in Alaska was likely a vagrant from New Mexico.

Other robust clades were in evidence among western individuals. Specimens from Texas, New Mexico, California, and Arizona consistently formed two small clades in all maximum likelihood analyses and shared unique haplotypes that were divergent from eastern samples and some western samples. These results are consistent with population fragmentation due to extensive degradation and loss of habitat that has occurred within the range of western Yellow-billed Cuckoos. Since the early 1900s, the Sacramento River Valley in California has experienced dramatic habitat loss as the human population has increased at a rate twice that of many developing countries (Mann and Plummer 1995). For example, massive destruction of habitat in the Buena Vista Lake area of Kern County and the Los Angeles Basin — which supported hundreds of cuckoos prior to 1900 — no longer contains any riparian vegetation (Laymon and Halterman 1991). Western populations are now seemingly isolated between human sprawl and agricultural lands in small pockets of

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remaining habitat in California, Arizona, New Mexico, Texas, and Mexico; all areas where they were once plentiful. These threatened populations comprise many small groups of individuals, potentially promoting local inbreeding that, over time, will be reflected in unique shared haplotypes (Zink et al. 2000) as demonstrated by my study.

In addition, specimens collected near Veracruz, Mexico (AY46908, AY46909) presented two unique fixed polymorphisms that were unlike any other samples used in my analyses. Banks (1988) also uncovered morphological differences characterizing Yellow-billed Cuckoos in Mexico. Furthermore, he suggested that populations in northeastern Mexico — Nuevo Leon, Tamalulipas, San Luis Potosi, and Zacatecas states in particular — represent a southeastern extension along the Gulf of Mexico coast from eastern Texas that is apparently isolated from other birds found further west in Chihuahua and Coahuila. The Veracruz specimens were collected outside of the known Yellow-billed Cuckoo breeding distribution so it is difficult to attribute them to either eastern or western populations; nonetheless, it is evident that cytochrome *b* was sufficient to delineate some genetic differentiation of these divergent Mexican populations.

Future Work

In this study, both ND2 and ND6 regions of the mtDNA genome showed no variation; thus, could not be used to define subspecies of Yellow-billed Cuckoo. On the other hand, cytochrome *b* showed significant haplotype variation within the western region, although it, too, was unable to clearly delineate between the eastern

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and western forms. This in no way suggests eastern and western Yellow-billed Cuckoo populations are not sufficiently divergent to be categorized as subspecies, simply that cytochrome *b* may not have the resolving power to distinguish between them. The question of cytochrome *b* resolving power is controversial, and its usefulness as a phylogenetic marker has been criticized in the past (Graybeal 1993; Meyer 1994). Moreover, several characteristics of cytochrome *b* can present obstacles for the phylogenetic algorithm, such as unequal base frequencies, rate inequalities, third position saturation, guanine deficiency, and insufficient variation at replacement sites (Yoder et al. 1996; Quinn 1997). In spite of these problems, cytochrome *b* remains a prevalent source of sequence data in avian studies, where it has taken a status as a universal metric whereby studies can be easily compared (Kvist 2000).

A gene with greater variability is obviously required to uncover the more recently evolved changes inherent to this subspecies question. This being the case, it is still unlikely that the appropriate gene would provide as clear-cut a resolution as the alleged four fixed base pairs of Pruett et al. (2001). Phylogenetics is rarely that straight-forward; subspecies designations are more often based on statistical analyses of haplotype variation and nucleotide diversity, not simply fixed variation within a subset of the data. Nevertheless, the answer may lie elsewhere on the mitochondrial genome. Genes used in this study represent mtDNA coding regions that are more conserved than non-coding regions because they actively code for specific proteins used by the organism. An amino acid substitution could be potentially deleterious; thus, sporadic mutation occurs less frequently, providing a

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gene more inclined to detect ancient changes at a resolution appropriate for order and family level studies. The purported results of Pruett et al. seemed unusual at onset, certainly enough to warrant my investigation of cytochrome *b* sequence variation in cuckoos. However, the control region, a non-coding region that mutates more freely, is likely more appropriate for providing an accurate measure of evolutionary relationships within and between subspecies and populations.

The study of the avian control region has been of growing interest in recent years, to address population structure, and to describe the organization and variation of the gene at several taxonomic levels (Roques, et al. 2004). The control region is responsible for the regulation of heavy (H) and light (L) strand transcription and of H-strand replication (Kvist 2000). It is the only large non-coding region in the avian mitochondria, and typically varies in size from approximately 1000 bp (e.g., 1044 bp in Muschovy Ducks, *Cairina moshata;* Liu et al. 1996) to 1200 bp (e.g., 1227 bp in Domestic Fowl, *Gallus gallus*: Desjardins and Morias 1990) in length. The central domain of the control region (Domain II) is most conserved, and contains several structural elements that can be readily aligned among bird families (Kvist 2000). In contrast, the other two domains of the control region (Domain I and Domain III) are highly variable, weak to selective constraints, and evolve very rapidly (Li 1997) and can often be used reliably to estimate the extent of divergence between populations and subspecies (Questiau et al. 1998; Young and Rhymer 1998; Barrowclough et al. 1999; Moulin et al. 2000; Scribner et al. 2003; Bowie et al. 2004). Therefore, sequencing and analyzing the entire control region may provide the correct level of resolution.

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In 2001, Fleischer attempted to characterize subspecific variation in Yellow-billed Cuckoos using the control region but was unable to arrive at a satisfactory conclusion. The fault may rest in the design of his analysis. First, he sequenced only part of the control region. He obtained a small 422 base pair fragment from "fresh" samples but relied only on a 255 base pair fragment derived from museum specimen toe pads dating from 1875 to 1955, which were likely highly degraded. Degraded DNA inherent in museum specimens frequently yields sequences only a few hundred base pairs in length. I acknowledge that obtaining samples from a rapidly declining species is difficult; however, my ability to acquire at least 19 western specimens, well representing the western distribution, demonstrates that it is not impossible. Sequencing the entire control region using recent tissue and/or blood samples may provide the necessary information to resolving the western Yellow-billed Cuckoo subspecific status question.

It is also recommended that further studies be conducted that investigated the role of DMSO damage in tissue samples: more specifically, attempts should be made to replicate DMSO damage in museum archived avian tissue. These studies must address many of several contributing factors, such as storage conditions, tissue type, volumes and concentrations of aqueous DMSO solutions, which all may have a role in the nature of degradation and damage in the DNA structure. Nonetheless our reliance on museum species will increase in future, particularly in light of the holistic decline in the world's natural biodiversity. It is imperative that we are confident in the ability of these precious specimens to reflect their evolutionary histories.

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Appendix 1. Complete list of Yellow-billed Cuckoo (*Coccyzus americanus*) and outgroup samples used in this study.

Taxon	Accession/Collection Number	State/Province	City/County
Coccyzus americanus	UAM 6953, CLD 399	Alaska	Juneau Quad
Coccyzus americanus	AMNH 7235, 381	California	Shasta
Coccyzus americanus	MH119-207555	California	Kern River
Coccyzus americanus	MH119-7040	California	Kern River
Coccyzus americanus	MH119-207553	California	Kern River
Coccyzus americanus	MH119-207576	Arizona	San Pedro River
Coccyzus americanus	MH119-207596	Arizona	San Pedro River
Coccyzus americanus	MH119-20742	Arizona	San Pedro River
Coccyzus americanus	MH119-207535	Arizona	San Pedro River
Coccyzus americanus	MH1212-13712	Arizona	San Pedro River
Coccyzus americanus	MH1212-13715	Arizona	San Pedro River
Coccyzus americanus	MH1212-13719	Arizona	San Pedro River
Coccyzus americanus	MH119-207594	Arizona	San Pedro River
Coccyzus americanus	NK11992, MSB 18032	New Mexico	Albuquerque
Coccyzus americanus	NK 103366, MSB 23134	New Mexico	Bitterlake NWR
Coccyzus americanus	NK 116168, MSB 23827	New Mexico	Socorro
Coccyzus americanus	NK 11964, MSB 18642	Texas	Atacosta NWR

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Appendix 1 continued.

Taxon	Accession/Collection Number	State/Province	City/County
Coccyzus americanus	LSUMZ B-37016	Texas	Hidalgo, Edinberg
Coccyzus americanus	LSUMZ B-23431	Texas	Jeff Davis
Coccyzus americanus	LSUMZ B-21785	Texas	Travis
Coccyzus americanus	UWBM 615, 2002-130	Louisiana	Cameron
Coccyzus americanus	UFNUMB 44090	Florida	Naples
Coccyzus americanus	UFNUMB 44087	Florida	Ft. Lauderdale
Coccyzus americanus	UWBM 78029, 2510	North Carolina	Macklenberg
Coccyzus americanus	AMNH 7466, Coll#619	New York	Suffolk County
Coccyzus americanus	UWBM 68159, 2000-091	Massachusetts	Barnstable
Coccyzus americanus	FMNH 441576	Wisconsin	Brown
Coccyzus americanus	FMNH 363714	Illinois	DuPage
Coccyzus americanus	FMNH 429371	Iowa	Marion
Coccyzus americanus	89937, MBR 2690	Kansas	Douglas
Coccyzus americanus	88591, MBR 27	Kansas	Jefferson
Coccyzus americanus	RAM Z01.3.1	Alberta	Edmonton
Coccyzus julieni	ANSP 4661	Ecuador	—

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Appendix 1 continued.

Specimen numbers refer to source collections: UAM, University of Alaska Museum, Fairbanks; AMNH, American Museum of Natural History, New York; NK, University of New Mexico, Albuquerque; FMNH, Field Museum of Natural History, Chicago; MBR, University of Kansas Natural History Museum, Lawrence; UF, Florida Museum of Natural History, Gainesville; UWBM, University of Washington Burke Museum of Natural History, Seattle; ANSP, Academy of Natural Sciences of Philadelphia; RAM, Royal Alberta Museum, Edmonton; LSUMZ, Louisiana State University Museum of Natural History, Baton Rouge; MH, Murrelet Halterman, University of Nevada, Reno. NWR, National Wildlife Refuge.

Appendix 2

Non-coding Region of the Mitochondrial DNA Genome in Cuckoos (Cuculidae)

The study of the avian control region, or D-loop, has been of growing interest in recent years to address population structure or subspecies divergence, using control region fragments, or to address questions at higher taxonomic levels through examination of the organization and variation of the entire region (Moulin et al. 2003; Roques et al. 2004). Although, the control region is considered to be the most variable region on the mitochondrial genome, the degree of variability differs among its three domains. Typically, left domain I is the most variable, the central domain II is the most conserved, and the right domain III intermediate between the two (Roques et al. 2004). Due to their higher degree of variability and reliability in estimating the rate of divergence, the first and third domains are used for most frequently in phylogenetic studies at the species and subspecies levels (Burg and Lomax et al. 2003; Friesen and Piatt 2003).

Desjardins and Morais sequenced the complete mitochondrial genome of a chicken (*Gallus gallus*) in 1990 and discovered that birds have a novel gene order compared to mammals and amphibians, in which ND5 is followed by Cytb, $tRNA^{Thr}$, $tRNA^{Pro}$, ND6, $tRNA^{Glu}$, and the control region in the 5' – 3' direction on the avian L strand (see Figure 14). The unusual order of genes surrounding the control region in the avian mtDNA genome has had an effect on sequencing for phylogenetic analyses. For example, Gibbs et al. (1996) examined the genetic differentiation of

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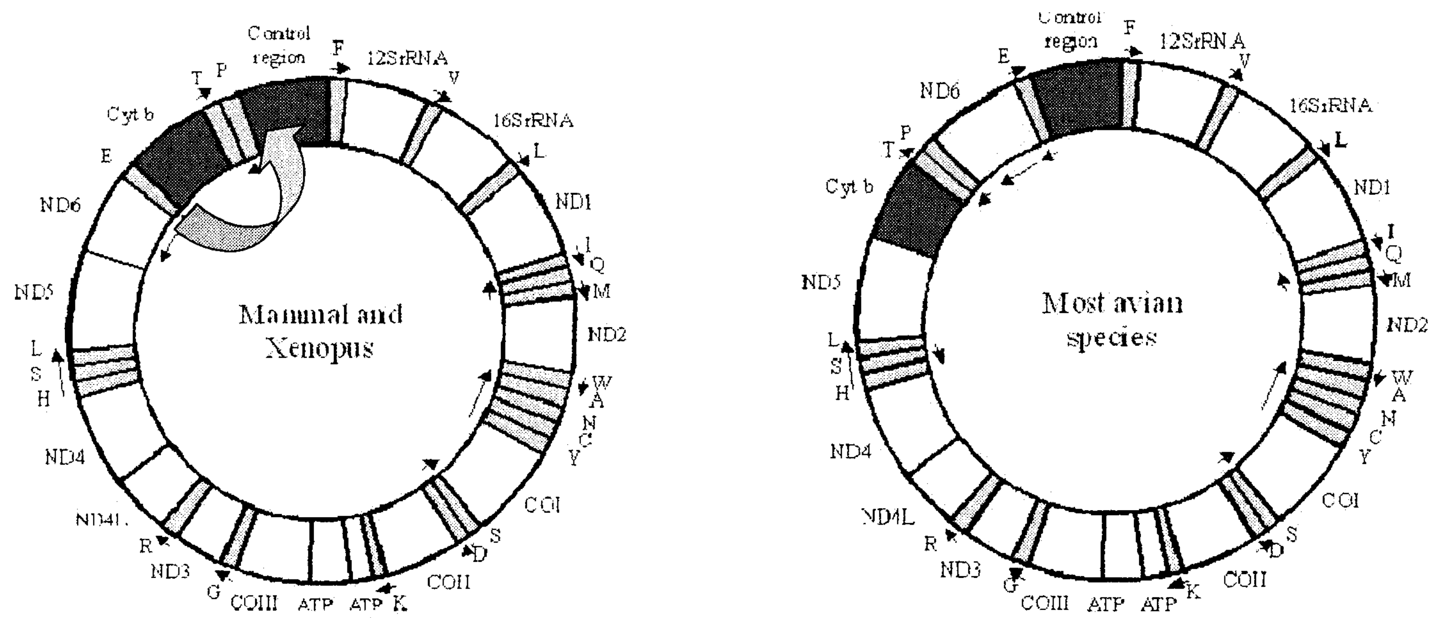
Figure 14. Mitochondrial DNA gene arrangement in birds (right) as compared to mammals and amphibians (left). In birds, ND5 is followed by cytochrome *b*, $tRNA^{Thr}$, $tRNA^{Pro}$, ND6, $tRNA^{Glu}$ and the Control Region in the 5'- 3' direction on the avian L strand. In birds, ND6 has translocated to the other side of cytochrome *b*, positioning itself next to $tRNA^{Glu}$ and the control region in the 5" direction, rather than being positioned between ND5 and cytochrome *b* (after Kvist 2000).



host races of Common Cuckoo (*Cuculus canorus*) using mitochondrial and microsatellite DNA variation. Their analysis focused on the control region as a target region due to the known high levels of intraspecific variation and suitability from population studies (Quinn 1992; Edwards 1993). To obtain a sequence that would allow the authors to develop cuckoo specific control region primers, they chose two previously-published primers that they thought would flank this entire region, thereby encapsulating the control region: ND6C-L (5' – CGA GAC AAC CCA CGG ACA AG 3'; Edwards 1993) situated towards the left hand region of the ND6 gene and GSC12S –H (5'- AAG GTT ACT AAG TCT TT –3'; Gelter *unpubl.*) located on the left hand region of the 12sRNA gene.

However, these authors were unaware of an additional novel mitochondrial gene order arrangement present in some birds. It has since been discovered that the Subocine Passeriformes, and orders Falconiformes and Cuculiformes, and family Picidae have a gene order configuration that differs from that of other birds, in which ND5 is followed by cytb, $tRNA^{Thr}$, control region, $tRNA^{Pro}$, ND6 and $tRNA^{Glu}$ in the 5' – 3' direction on the avian L strand (Mindell et al. 1998; see Figure 15). This gene arrangement is thought to have multiple independent origins, as it was found in a number of divergent taxa. As a result, the fragment amplified by Gibbs et al. (1996) was not the control region but, rather, the non-coding region — typically about 300 base pairs (bp) in length — that is located between $tRNA^{Glu}$ and $tRNA^{Phe}$, which is further flanked on either side by ND6 and 12sRNA in the 5'–3' direction.

Gibbs et al. (1996) remarked that their analysis showed that the cuckoo had an unusually short control region approximating 300–450 bp that also possessed an

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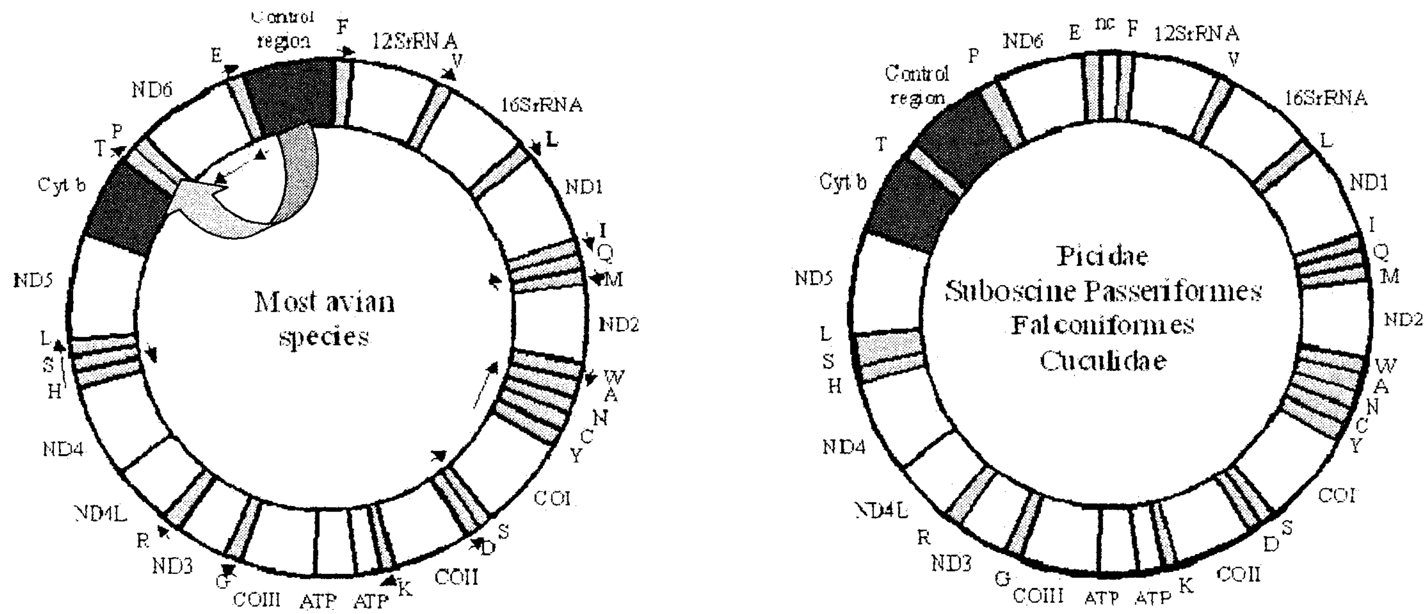
Figure 15. Novel gene order of Cuculidae, Falconiformes, Subocine Passeriformes, and Picidae (right) as compared to the typical gene order exhibited by most avian species (left). In novel gene order species, the Control Region has translocated in the 3' – 5' direction, and is positioned between $tRNA^{Thr}$ and $tRNA^{Pro}$. Thus, the control region is flanked by cytochrome *b* and ND6 in the 5'-3' direction, rather than being positioned between ND6 and 12s (after Kvist 2000).



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unusual sequence organization, having a 174 bp of single copy sequence followed by 20-36 tandem repeat copies of the motif CAACAAA. Most avian control regions sequenced to date are between 1000 and 1500 bp (Baker and Marshall 1997; Ruokonen and Kvist 2002), but can be much longer; such as 1758 bp in Adelaide Penguins (Ritchie and Lambert 2000) and 2040 bp in Little Blue Penguin (Slack et al. 2003; Pereira et al. 2004). In these cases, length variation is due primarily to insertion or deletion of a few nucleotides and varying numbers of tandem repeats or microsatellites. Tandem repeat sections, varying in length from 79 bp to 49 bp (repeated 9, 12, or 13 times), or 7 bp repeated 13, 14, 18, or 20 times, are often found in avian control regions (Pereia et al. 2004). The sequence similarity between this non-coding region and the control region suggests a duplication event involving at least part of the control region, and the variable length of non-coding regions found in avian species may be remnants of a gene rearrangement process involving the control region (Mindell et al. 1998).

These results were verified when I sequenced this target region using the primer set in Gibbs et al. (1996). Further analysis of the data revealed the tandem repeat section of the non-coding region, the complete ND6 gene, and first few hundred base pairs of 12sRNA. This non-coding tandem repeat section was also present at the 5' end of the control region sequenced in this study, adding further support for the rearrangement or duplication of part of the control region into the non-coding of the avian mtDNA genome. Later, Gibbs et al. (2000) determined the correct sequence variation in a 411 bp portion of the left hand hypervariable domain

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I region of the control region using two *Cuculus*-specific primers designed in the study, but did not sequence the entire genome (domains II and III).

Until now, the actual size of the control region in a Cuculidae species was not definitively known. To date, I have successfully amplified and sequenced the entire control region for 31 samples of Yellow-billed cuckoo (*Coccyzus americanus*) I have found the control region in this species to be 1100 bp in length.

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