

## AN ABSTRACT OF THE THESIS

Dacey M. Mercer for the degree of Master of Science in Wildlife Science presented on September 25, 2008.

Title: Phylogeography and Population Genetic Structure of Double-crested Cormorants (*Phalacrocorax auritus*).

Abstract approved:

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Susan M. Haig

The Double-crested Cormorant (*Phalacrocorax auritus*) is a widespread, colonial, North American waterbird with bicoastal and inland distributions. Four subspecies have been described within North America corresponding to five geographic breeding regions: Interior and North Atlantic cormorants (*P. a. auritus*); Southeastern cormorants (*P. a. floridanus*); Alaskan cormorants (*P. a. cincinnatus*); and Pacific cormorants (*P. a. albociliatus*). Management strategies vary widely across the species' range according to local perceptions rather than relative population status. An understanding of population genetic structure is necessary for delineating appropriate management units.

We examined the genetic structure of Double-crested Cormorants across their range in the United States and Canada to quantify variation within and among breeding sites and to assess the status of traditional geographically defined subspecies. Sequences (700bp) from domains I and II of the mitochondrial control region were analyzed for 234 Double-crested Cormorants from 23 breeding sites. Variation was also examined at 8 microsatellite loci for 395 cormorants from the same 23 breeding sites. The mtDNA and

microsatellite data provided strong evidence that the Alaskan subspecies is genetically divergent from other populations in North America (net sequence divergence = 6.72%;  $\Phi_{ST}$  for mtDNA control region = 0.738;  $F_{ST}$  for microsatellite loci = 0.05). Our data also suggested strong genetic divergence in the southwestern U.S.; southern California may represent a zone of introgression resulting from a northward expansion of a unique lineage from the species' range in northwestern Mexico. In contrast, there was little support for recognition of subspecies within the conterminous U.S. and Canada, outside of Alaska. Rather than genetically distinct regions corresponding to the putative subspecies, we observed a distribution of genetic variation consistent with a pattern of gradual isolation by distance. This pattern implies that genetic differences across the range are due to geographic distance rather than discrete subspecific breaks. Although three of the four subspecies were not genetically distinct, potential demographic separation, habitat differences, and recent declines at some colonies within the regions, suggests that the Pacific and possibly the North Atlantic breeding regions may still warrant consideration as distinct populations.

This thesis provides the first species-wide assessment of the phylogeography and population genetic structure of the Double-crested Cormorant. It further resulted in the first microsatellite markers developed specifically for a North American pelecaniform. The mitochondrial and microsatellite data provide a comprehensive assessment of the four putative subspecies described for the species. Given the highly varying conservation status of Double-crested Cormorants throughout their range, results of this study provide guidance for conservation and management practices on their behalf in North America.

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Phylogeography and Population Genetic Structure of Double-crested Cormorants  
(*Phalacrocorax auritus*)

by  
Dacey M. Mercer

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APPROVED:

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Major Professor, representing Wildlife Science

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Head of the Department of Fisheries and Wildlife

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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Dacey M. Mercer, Author

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## CONTRIBUTION OF AUTHORS

Dr. Susan Haig and Dr. Daniel Roby were involved with study design, project completion, and writing of manuscripts, particularly Chapter 3. Thomas Mullins was involved with development and implementation of laboratory methodologies and writing of Chapter 2.

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# **Phylogeography and Population Genetic Structure of Double-crested Cormorants (*Phalacrocorax auritus*)**

## **Chapter 1. Introduction**

Contemporary conservation biologists are faced with the difficult challenge of designing strategies to protect and manage biodiversity in the face of serious anthropogenic perturbations. With this challenge comes a need for methods that can identify units for conservation and management purposes, particularly units below the species level (Fraser and Bernatchez 2001). Conservation genetics is a subdiscipline of conservation biology well-suited to address this problem. The fundamental aim of conservation genetics is to identify and characterize patterns of genetic diversity at multiple levels to help preserve options for future evolution, and to provide tools that will aid in conservation and management towards this end (Groom 2006). This new field draws upon evolutionary, population, and quantitative genetics, as well as taxonomy (Frankham et al. 2002). Additionally, the ability of molecular techniques to describe relationships among individuals, populations, and species has increased so that molecular analyses can now resolve population structure at multiple spatial scales.

Identification of intraspecific units was originally based on taxonomically recognized subspecies, primarily determined by differences in phenotypic traits and geographic distribution (Haig et al. 2006). Mayr et al. (1953) described subspecies as “geographically defined aggregates of local populations which differ taxonomically from other such subdivisions of the species” and thus recognized geographic races as formal taxonomic units. The assumption is that when secondary characters vary geographically,

the variation tends to follow whatever diagnostic characters are chosen to delimit races and with further analysis subspecies can be shown to be discrete units (Wilson 1953). Further, conservation efforts have often been aimed at the subspecies level on the premise that subspecific variation represents local adaptation (Zink 2004).

Critics of the application of subspecies as conservation units have noted that there are several deficiencies in this approach, including the uneven application of subspecies designations across taxa, confusion among subspecies criteria, and frequent disagreements between historical taxonomic subdivisions and those revealed by molecular assays (Haig et al. 2006). The fundamental criticism of most currently recognized subspecies is that the geographic variation in morphology used to define them may not reflect underlying genetic structure and phylogenetic relationships (Zink 2004). This has proven to be the case in several avian studies where population structure based on genetic variation disagreed with subspecies defined by morphology (Ball & Avise 1992; Zink et al. 2000; Zink 2004). For example, Ball and Avise (1992) found that among 16 traditionally recognized subspecies, only two proved readily distinguishable in comparison with conspecifics by mtDNA haplotypes. Additionally, in a study of 41 species Zink (2004) found that only 3% of avian subspecies qualify as distinct evolutionary entities according to genetic criteria, although biological species of birds did contain 1.9 independently evolving groups on average, irrespective of the number of named subspecies. In contrast, many recent genetic studies have found support for traditional avian subspecies (Randi et al. 2003; Haig et al. 2004; Funk et al. 2007; Funk et al. 2008; and others).

Despite criticisms and debate over criteria used to define subspecific units, interest in intraspecific population structure and delineation of conservation units has heightened as a result of increasing policy and legislation that allows for conservation and management below the species level. Most notably, the U.S. Endangered Species Act (ESA) of 1973 allowed for protection of species and subspecies, resulting in the listing of numerous taxa of subspecific rank. Additionally, non-taxonomic subdivisions of species are now recognized by some conservation legislation and these concepts have become the subject of much attention and debate. In particular, the U.S. Endangered Species Act (ESA) was amended in 1979 to extend the authority to list to “any distinct population segment of any species of vertebrate fish or wildlife that interbreeds when mature” (U.S. Congress and Senate 1979). This amendment addressed the need for greater flexibility in endangered species legislation and allowed for differential management practices for populations of the same species, but did not provide a specific definition for distinct population segment (DPS). The Fish and Wildlife Service (USFWS) and the National Marine Fisheries Services (NMFS) have since adopted a policy to clarify their interpretation of a DPS as being markedly separated from other populations of the same taxon, as evidenced by genetic, ecological, or morphological discontinuities, and showing biological or ecological significance (USFWS and NMFS 1996). In addition, the criterion for identification of DPSs was extended to use of the evolutionarily significant unit (ESU) concept in Pacific salmon (Waples 1995; USFWS and NMFS 1996).

In accordance with the aim of identifying conservation units, increasing the extent and quality of data available on distributions of communities, species, and genetics

increases our ability to develop strategies for conservation and management (Moritz 2002). By quantifying and apportioning total genetic diversity within and among populations, the spatial distribution of variation can be examined and used to define areas of conservation interest (Groom et al. 2006). Recent and rapid advances in molecular methodologies and statistical analyses have aided this pursuit, providing for more rigorous examination of genetic structure and testing of theories in population genetics and phylogeography (Avisé 2000).

Mitochondrial DNA (mtDNA) and microsatellite DNA are genetic markers that have been used to look at phylogeny, phylogeography, and population structure in avian species (Wenink et al. 1994; Zink et al. 2000; Abbot and Double 2003a; Randi et al. 2003; Sgariglia and Burns 2003; and others). MtDNA has a simple genetic structure and a matrilineal mode of inheritance without rearrangement or recombination (Avisé 2004). This mode of inheritance results in an effective population size one fourth that of nuclear alleles. The subsequent high rates of haplotype extinction (Avisé 2004) coupled with high rates of mutation, makes mtDNA sequence data useful for inferring intraspecific phylogeography (Avisé 1987). Conversely, a microsatellite is a short section of tandemly repeated DNA sequence within the nuclear genome of an organism, with the number of repeats at a particular locus being hypervariable (highly polymorphic) between individuals of the same species (Avisé 2004). In contrast to mtDNA, microsatellites are biparentally inherited nuclear markers that estimate gene flow mediated by both sexes, and can be used to distinguish between individuals and more clearly examine fine-scale population structure (Paetkau et al. 1998; Abbott and Double 2003b).

The ability to describe population genetic architecture and identify and order the biological forces responsible for the structure is a continuing challenge (Avice 2004). Genetic structure may be influenced by many forces, including: migration or gene flow, random genetic drift, natural selection, and numerous ecological and life history factors of the particular organism. Avian taxa present an interesting case as they tend to be highly vagile with large geographic distributions, suggesting high gene flow, but may also exhibit strong natal philopatry, indicating low inter-population gene flow (Avice 2004). In fact, some avian species have shown little or no mtDNA phylogeographic structure across much of North America, while other species have displayed pronounced mtDNA phylogeographic structure at varying spatial scales (reviewed in Avice 2000).

In conclusion, it can not be assumed that existing nomenclature accurately describes patterns of genetic diversity or that recognized subspecies represent evolutionary entities. Identification of population structure, through genetic analysis, can be more useful than current subspecific designations for directing appropriate conservation and management (Zink 2004). Molecular methods can readily sample groups and individuals over large geographic distances, whereas traditional methods (mark/recapture, radio telemetry, or satellite telemetry) are often prohibitively labor intensive, expensive, or spatially limited (Haig et al. 1998, Webster et al. 2002). Population genetic and phylogenetic analyses can then resolve population genetic structure and identify conservation units. The detection of genetic structure and correlation with landscape can identify natural population subdivisions and help with the

understanding of demographic and evolutionary patterns within populations, providing information that is crucial for appropriate conservation and management.

Double-crested Cormorants (*Phalacrocorax auritus*) are large, North American, colonial waterbirds characterized by a long neck, hooked bill, and habit of sitting with wings outstretched to dry, as feathers are not fully waterproof (Hatch and Weseloh 1999). They occupy diverse aquatic habitats including coastal waters, estuaries, lakes, rivers, ponds, and lagoons (Palmer 1962). Colonies are highly variable in size, ranging from a few (rarely a single pair) to several hundred thousand pairs (Bent 1922; Palmer 1962). They may be located on rocky or sandy islands, man-made structures such as bridges, docks, and transmission line towers, or in trees and shrubs near water (Chapdelaine and Bedard 1995; Stenzel et al. 1995; Hatch and Weseloh 1999). A clutch of 1-7 eggs (modal clutch 4), laid in a rudimentary stick constructed nest, is incubated and cared for by both parents. Fledging success is typically 1.2-2.4 young/nest (Palmer 1962; Hatch and Weseloh 1999). Double-crested Cormorants usually feed close to shore in shallow open water and dive from the surface to pursue prey underwater. Diet is almost entirely fish and generally slow-moving or schooling species ranging in size from 3-40 cm (Wires et al. 2001). They are opportunistic, however, taking a wide range of prey species depending on availability (Hatch and Weseloh 1999).

Information about population connectivity of Double-crested Cormorants throughout the annual cycle is limited. Birds nesting in the interior and on the Atlantic Coast are highly migratory, while birds in other regions are mostly resident within their breeding range (Hatch and Weseloh 1999). The birds breeding east of the Rocky



Mountains migrate mainly to Florida and the Gulf of Mexico (Dolbeer 1991), while those breeding in the mountain states are thought to migrate to the west coast. Despite migratory tendencies, mate selection and pairing occurs at the breeding colony and such a system is conducive to high natal philopatry in stable colonies (Hatch 1995). In fact, recoveries of banded birds have indicated high philopatry to areas near the natal colony (Dolbeer 1991). Many young birds first breed where they were hatched and new colonies are thought to be formed by young birds nesting in the closest suitable habitat to the natal colony (Hatch 1995). New colony sites may be abandoned within a few years, but once established are likely to persist (Hatch and Weseloh 1999).

The Double-crested Cormorant is the most widespread of the six species of cormorant (Family Phalacrocoracidae) in North America, occurring on both coasts and inland (American Ornithologists' Union; AOU 1998). The species is most commonly described as consisting of five more-or-less discrete geographic breeding regions (Hatch 1995; Hatch and Weseloh 1999; Tyson et al. 1999; Wires and Cuthbert 2006). These regions directly correspond to the four traditional subspecies, with the nominate subspecies comprised of two regional populations (Bent 1992; Palmer 1962; American Ornithologists' Union (AOU) 1957). The general geographic distributions are as follows: *P. a. auritus* occupies the Interior and the North Atlantic regions, and has the widest subspecies distribution across northeastern and central North America; *P. a. floridanus* occupies the Southeast region, and breeds from Texas to Florida and north to the Carolinas; *P. a. cincinnatus* occupies the Alaska region, and breeds solely in Alaska;

*P. a. albociliatus* occupies the Pacific region, and breeds along the Pacific Coast from British Columbia south to Sinaloa, Mexico and inland, possibly to New Mexico, Utah, and Montana (Hatch 1995). More recently, a fifth subspecies (*P. a. heuretus*) was described by Watson et al. (1991) in the Bahamas. These subspecies were primarily based upon allopatry of breeding regions. The geographic distributions are not entirely discrete, however, but overlap and are poorly defined in some locations (Hatch 1995). Subspecies were also differentiated by size and crest character, but there is considerable variation in morphology, the distribution of crest characters is poorly known, and no significant phenotypic differences between subspecies have been characterized. In general, average size is described as increasing from south to north along both coasts and from east to west, and plumage varies across regions (Palmer 1962). Specifically, southeast birds are smallest and darkest with black crests, northeast birds have occasional white or partially colored crests, and north Pacific birds are largest and often have all-white crests (Palmer 1962).

The Double-crested Cormorant recently experienced two distinct periods of population decline followed by subsequent rebounds in population numbers and current explosive growth in some portions of its range. The initial decline occurred in the late 19<sup>th</sup> century and early 20<sup>th</sup> century, with extirpation from parts of the range such as New England, Vancouver Island, islands of northwest Baja Californian islands (including Isla San Martin), and the western Aleutian islands, primarily due to direct persecution (Hatch and Weseloh 1999). The second drastic population decline occurred in the latter half of the 20<sup>th</sup> century and was primarily attributed to increased mortality from pesticide

contamination (Hatch and Weseloh 1999). Interior populations reached a low point in the 1970's and North Atlantic populations ceased growing (Hatch 1995). Numbers began increasing about 1975, with Interior populations experiencing explosive growth from 1975-1995 and subsequent range expansion (Tyson et al. 1999). This rapid increase has been attributed to decreased pesticide-related mortality resulting from the ban of DDT in 1972; increased protection afforded by the addition of the Double-crested Cormorant to the Migratory Bird Treaty Act in 1972; and human-induced changes in the aquatic environment, including changes in fisheries, development of aquaculture in the south, and creation of additional breeding and foraging habitats (e.g., dredge spoil islands, reservoirs; Wires et al. 2001).

Although most populations exhibited growth by the 1980's, the largest growth rate and greatest increase in number was restricted to the Interior (Hatch and Weseloh 1999). The most dramatic increase occurred in the Great Lakes area, with numbers increasing from about 38,000 in 1990 to roughly 93,000 pairs in 1997 (Tyson et al. 1999). In contrast, the Alaska, North Atlantic, and parts of the Pacific populations exhibited negative rates of change from 1990-1994 (Tyson et al. 1999), with colony declines documented over much of southern Alaska, British Columbia, Washington, and southern California (Carter et al. 1995; Hatch and Weseloh 1999; Anderson et al. 2004). Growth of the North Atlantic population (Nova Scotia to Massachusetts) may have ceased by 1990 (Hatch and Weseloh 1999), the Pacific population has yet to reach pre-pesticide numbers (Wires and Cuthbert 2006), and the Alaskan population is likely still experiencing declines (D.B. Irons, pers. comm.). The observed colony declines may be

related to reduced suitability of colony sites associated with increased human disturbance in the Atlantic (Krohn et al. 1995), and habitat loss, pollution, human disturbance, and introduced predators in Alaska and along the west coast (Carter et al. 1995). A recent assessment of the Pacific subspecies, *P. a. albociliatus*, (USFWS, unpubl. data) indicates a gradual continued increase in total population size; however, the distribution of the subspecies across its breeding range has changed dramatically and colony declines continue over much of British Columbia and parts of California (Moul and Gebauer 2002; Anderson et al. 2004; Capitolo et al. 2004; USFWS unpubl. data). Currently, nearly 50% of the Pacific population now nests in only a single colony located on East Sand Island, in the Columbia River estuary, Oregon (Anderson et al. 2004, Roby et al. 2008).

The Double-crested Cormorant is generally described as over-abundant and often perceived as a nuisance species. Recent counts estimate the total population to be 1-2 million individuals (about 350,000 breeding pairs), with large variation among subspecies (Hatch 1995). Numbers of breeding pairs in each subspecies were estimated as: *P. a. auritus*: 257,000-357,000 pairs (Interior: 170,000-270,000 pairs; North Atlantic: 87,000 pairs); *P. a. floridanus*: 9,400 pairs (Southeast); *P. a. albociliatus*: 33,000 pairs (Pacific); *P. a. cincinnatus* 3,029 pairs (Alaska; Wires and Cuthbert 2006). Additionally, the Bahaman population (*P. a. heuretus*) was estimated as only 212 pairs (Hatch 1995). Recent analysis of historical records however, suggests historical abundance has not been attained by current Double-crested Cormorant populations (Wires and Cuthbert 2006), and most of the modern expansion represents recolonization of the historical range (Hatch and Weseloh 1999).

The Double-crested Cormorant has gone from being a species of concern in 1970, warranting 'Endangered' status in several states, including Wisconsin, Illinois, and Michigan, to being perceived as a pest requiring population control (Weseloh et al. 1995). In the U.S., the species has been protected by federal law since 1972, when cormorants were added to the list of migratory bird families protected by the U.S.-Mexican Migratory Bird Convention in an amendment to the 1916 Migratory Bird Treaty Act (Trapp et al. 1995). However, shooting and destruction of nests, eggs, and young has long occurred and such activities still continue on a large scale in some areas. Increases in numbers since the 1980's have further enhanced cormorant conflicts on three main fronts: (1) perceived competition with fisherman, (2) depredation at commercial aquaculture facilities (primarily catfish farms in the southeastern U.S.) and, (3) alteration of vegetation and nest trees resulting in impacts on other colonial waterbirds (Duffy 1995; Hatch and Weseloh 1995). Large-scale management plans to reduce cormorant numbers have been implemented in several areas, including: New England (Krohn 1995), the Great Lakes (Weseloh et al. 1995), and the St. Lawrence River (Bedard 1995). In addition, the USFWS has issued increasing numbers of depredation permits in recent years for control of fish-eating birds at aquaculture facilities in the southeastern states (Trapp et al. 1998). In March 1998, the USFWS established a depredation order that allows aquaculturists to shoot cormorants without a federal permit in 13 eastern states (Trapp et al. 1998). In Canada, cormorants are protected and managed under provincial, rather than federal, law with variable local practices including strict protection, individual kill permits, government-managed cull, and large illegal kills (Keith 1995). Notably, in

British Columbia the species is designated as “threatened” on the provincial Red List (British Columbia Conservation Data Centre 2003).

Recently, increased concerns over possible impacts to natural resources due to burgeoning cormorant populations prompted the USFWS in conjunction with the U.S. Dept. of Agriculture/Wildlife Services (USDA/WS) to prepare an Environmental Impact Statement (USDI/FWS 2003a). The final published rule established a Public Resource Depredation Order for Double-crested Cormorants effective in 24 eastern states, and revised the 1998 Double-crested Cormorant Aquaculture Depredation Order to include lethal control at winter roost sites (USDI/FWS 2003b). Double-crested in the western U.S., however, were not included in the Environmental Impact Statement or Depredation Order and are not actively managed for population control or conservation. Thus, management efforts for this species continue to vary across North America and are primarily influenced by local population status and perception of its role as a nuisance species.

As yet, no comprehensive genetic work has been conducted to confirm or reject subspecific designations in the Double-crested Cormorant. The only genetic studies of the species to date exclusively examined the relationship between southeastern U.S. colonies and northeastern U.S. colonies (Waits et al. 2003, Green et al. 2006). These studies failed to find significant genetic structure of mitochondrial haplotypes (Waits et al. 2003) or microsatellites (Green et al. 2006), but both studies were limited by small sample sizes of populations and individuals. Green et al. (2006) did find high levels of microsatellite variation and statistically significant genetic differentiation among some sampled sites.

Waits et al. (2003) found little variation in five mitochondrial gene regions, including the control region (355bp), 12sRNA (414bp), CO3-ND3 (461bp), cytochrome b (342 bp), or 16s RNA (992 bp). It is not surprising that conservative regions of the mtDNA, such as CO3-ND3, 16S, and 12S exhibited no differentiation among populations. However, further examination of the variable control region, or the less conserved ND6, may reveal greater mitochondrial variation. In contrast, microsatellite analysis of the Great Cormorant (*P. carbo*) revealed small, but significant differences among populations across Europe, with greater levels of population divergence between the putative subspecies (Goostrey et al. 1998). This species is similar to the Double-crested Cormorant in that it comprises six taxonomically recognized subspecies that have experienced population changes like those of the Double-crested Cormorant.

Research presented in this thesis represents a thorough genetic analysis of the Double-crested Cormorant within North America. Phylogenetic and population genetic analyses were conducted to describe the genetic variation and patterns of genetic differentiation within and among cormorant populations. Individual chapters were prepared as manuscripts for submission to peer-reviewed journals. Chapter 2 describes the development and characterization of six novel microsatellite loci for the Double-crested Cormorant. Chapter 3 describes phylogeographic and population genetic structure of the species and evaluates the status of traditional geographic subspecies. Results provide an understanding of population structure and status that will aid management and conservation.

## Chapter 2. Isolation and characterization of six novel microsatellite loci in the Double-crested Cormorant (*Phalacrocorax auritus*)

### ABSTRACT

We describe the isolation and characterization of six microsatellite loci from the Double-crested Cormorant (*Phalacrocorax auritus*) using a hybridization capture approach. All loci were variable with the number of alleles ranging from 2 to 17, and heterozygosity ranging from 0.132 to 0.918. No loci showed signs of linkage disequilibrium and all loci, except DCCO-06, conformed to Hardy-Weinberg equilibrium frequencies. In addition, all loci successfully amplified and were polymorphic in two related *Phalacrocorax* species. These loci should prove to be applicable to studies of phylogeography and population genetic structure in the Double-crested Cormorant and other members of the genus *Phalacrocorax*.

The Double-crested Cormorant (*Phalacrocorax auritus*) is a widespread, colonial, North American waterbird with bicoastal and inland distributions (Hatch and Weseloh 1999). The species is commonly described as consisting of four subspecies (although see Mercer et al., in prep: Ch. 3): *P. a. auritus* has the widest subspecies distribution across northeastern and central North America; *P. a. floridanus* breeds from Texas to Florida and north to the Carolinas; *P. a. cincinnatus* breeds solely in Alaska; *P. a. albociliatus*



breeds along the Pacific Coast from British Columbia south to Sinaloa, Mexico and inland, possibly to New Mexico, Utah, and Montana (Hatch and Weseloh 1999).

Management efforts vary widely across the species range according to local perceptions rather than relative population status. An understanding of population genetic structure is necessary for delineating appropriate management units.

The *Phalacrocorax* is a genetically under-studied genus in a genetically under-studied order, Pelecaniformes. Consequently, there is a pronounced lack of molecular markers developed for cormorants. The only microsatellite loci previously developed for a member of the *Phalacrocorax* were isolated from the Great Cormorant (*Phalacrocorax carbo*; Piertney et al. 1998) of Europe. Here we describe the isolation and characterization of six variable dinucleotide and tetranucleotide microsatellite markers which will be suitable for examination of local and regional population structure and gene flow within the Double-crested Cormorant.

Microsatellite markers were developed using an enrichment protocol (Glenn and Schable 2005). Approximately 2 mg of genomic DNA (gDNA) from one individual was digested with *Bst*I and *Xmn*I, and SuperSNX24 linkers (F: GTTTAAGGCCTAGCTAGCAGAATC, R: GATTCTGCTAGCTAGGCCTTAAACAAAA) were ligated onto the ends of gDNA fragments. Five biotinylated di-, tri-, and tetra-nucleotide probes ((AG)<sub>12</sub>, (TG)<sub>12</sub>, (AAC)<sub>6</sub>, (AAT)<sub>12</sub>, and (TCAC)<sub>6</sub>) were hybridized to gDNA. The biotinylated probe-gDNA complex was added to streptavidin magnetic beads (Dynabeads® M-280, Invitrogen) to enrich for microsatellite containing fragments. This mixture was washed twice with 2x SSC, 0.1% SDS and four times with 2x SSC, 0.1% SDS at 52 °C. For the

final two washes, the mixture was incubated for 1 min in a 52 °C water bath. Between washes, a magnetic particle-collecting unit was used to capture the magnetic beads, which are bound to the biotin-gDNA complex. Enriched fragments were eluted from the biotinylated probe by denaturing in TLE at 95 °C and precipitated with 95% ethanol and 3 M sodium acetate. To increase the amount of enriched fragments, a 'recovery' PCR was performed on a PTC 100 thermal cycler (MJ Research) in a 25µl reaction containing 10x PCR buffer, 1.5 mM MgCl<sub>2</sub>, 2.5 mM of each dNTP, 25 µg/ml BSA, 0.50 µM of the SuperSNX24 forward primer, 1 U *Taq* DNA polymerase, and approximately 25 ng of the enriched gDNA fragments. Thermal cycling was performed as follow: 95 °C for 2 min followed by 25 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 90 s, and a final elongation step of 72 °C for 30 min. Subsequent PCR fragments were cloned using the TOPO-TA Cloning® kit following the manufacturer's protocol (Invitrogen). Bacterial colonies containing a vector with gDNA were used as a template for subsequent PCR in a 25µl reaction containing, 10x PCR buffer, 2 mM MgCl<sub>2</sub>, 10 µM of each dNTP, 25 µg/ml BSA, 10 µM of the M13 primers, and 1 U *Taq* polymerase. Thermal cycling was as follows: an initial denaturing step of 95 °C for 3 min was followed by 35 cycles of 95 °C for 20 s, 50 °C for 20 s, and 72 °C for 90 s. PCR amplification quality was assessed by visualizing 5µl of the product with ethidium bromide on 1% agarose gels. Sequences were generated using ABI Prism Big Dye Terminator Cycle Sequencing chemistry on an ABI 3100 capillary DNA automated sequencer (377 DNA Sequencer ABI Prism 377XL Collection Software) located in the Central Services Laboratory at Oregon State University. We designed primer pairs to amplify 40 different loci from the resulting

sequences. Among these loci, six produced successful PCR amplifications and were polymorphic for the Double-crested Cormorants analyzed (Table 2.1).

DNA was extracted from blood or tissue samples of 395 apparently unrelated Double-crested cormorants from 23 breeding sites across North America using a standard phenol/chloroform extraction. Briefly, 10  $\mu$ l of blood or 1 mm<sup>3</sup> of tissue was digested in 400  $\mu$ l of extraction buffer (50 mM Tris, pH 8.0; 10 mM EDTA, pH 8.0; 200 mM NaCl; 2% SDS) with Proteinase K (20 mg/ml) added to a final concentration of 600  $\mu$ g/ml. Samples were then vortexed and incubated overnight (~18 h) at 50 °C. Samples were extracted with equal volumes of phenol (saturated with 10mM Tris, pH 8.0) and then chloroform/isoamyl alcohol (25:1). DNA was cleaned and concentrated by centrifugation dialysis using Microcon 30,000 MW cutoff columns (Amicon Bioseparations). Stock DNA was diluted to a concentration of 100 ng/ $\mu$ l for experimental use.

Amplifications were performed using a PTC 100 thermal cycler (MJ Research). A total reaction volume of 20  $\mu$ l was used with the following concentrations: 10 mM Tris-HCl at pH 8.3; 50 mM KCl; 0.001% gelatin; 3.5 mM MgCl<sub>2</sub>; 100  $\mu$ M for each of the dNTPs; 0.2  $\mu$ M of each primer; 100 ng of template; and 1.5 U AmpliTaq Gold Polymerase (Perkin Elmer). The following parameters were used for amplifications: 12 min denaturation at 93 °C, followed by 35 cycles of 30 s at 93 °C, annealing at 54 °C for 30 s, and elongation at 72 °C for 1 min. A final 10 min elongation period at 72 °C followed the last cycle. PCR amplification quality was assessed by visualizing 5  $\mu$ l of the product with ethidium bromide on 1% agarose gels. Amplification products were analyzed on an ABI 3100 capillary DNA automated sequencer located in the Central

Services Laboratory at Oregon State University. ABI Genescan<sup>®</sup> analysis was used to determine the size of fragments based on internal lane standard GeneScan 500 [Rox]. ABI Genotyper<sup>®</sup> software was used to score alleles accurately.

MICRO-CHECKER software (Oosterhout et al. 2004) was used to check for the presence of null alleles and to assess the presence of other genotyping errors, such as nonamplified alleles, short allele dominance, and scoring of stutter peaks. Linkage disequilibrium between loci across all sites and deviations from Hardy-Weinberg genotype frequency for each loci and site were tested with GENEPOP version 3.4 (Raymond and Rousset 1995). For all these analyses, significance was evaluated by Fisher exact tests where  $P$  values were estimated by applying a Markov chain method. Sequential Bonferroni corrections were applied to determine the significance level of each test and correct for type I error (Rice 1989).

The six loci examined were polymorphic and consistently amplified clean, unambiguous peaks (Table 2.1). The number of alleles per locus ranged from 2 to 17 and the average gene diversity,  $H_E$ , per locus ranged from 0.132 to 0.918. No significant linkage disequilibrium was detected among the six loci. The only locus that deviated significantly from Hardy-Weinberg equilibrium was locus DCCO-06. Null alleles may be present at this locus, as indicated by MICRO-CHECKER version 2.2.3 (Oosterhout et al. 2004). These loci should prove highly informative for population genetic studies, with the exception of possibly eliminating locus DCCO-06.

We then tested for cross-species amplification in two additional cormorant species: Brandt's Cormorant (*P. penicillatus*) and Pelagic Cormorant (*P. pelagicus*). The

same PCR conditions and electrophoretic methodology were used as previously described. All primer pairs resulted in successful amplifications and gave a single PCR product of expected size that was polymorphic, suggesting that these primers could be useful for population studies in related species.

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**Table 2.1** Summary statistics of microsatellite loci isolated from the Double-crested Cormorant genotyped on 395 individuals from 23 breeding sites throughout North America. Primer sequences are given in the 5'-3' orientation together with the optimal PCR annealing temperature, fragment lengths (bp), number of alleles resolved, and average gene diversity ( $H_E$ ).

Locus	Repeat Motif	Primer Sequence (5'-3')	Anneal T.	Length	No. alleles	$H_E$
Dcco-01	(TCAC) <sub>19</sub>	F-GCTCAGTTGCTTTTCTCAC R-TTGCTGAATGGCTGTTCCA	54	191-251	13	0.87
Dcco-02	(TG) <sub>20</sub>	F-TAAAGCTAGGGTGATGGGC R-GCTGGAAATCTGGAAATCTCT	54	222-256	17	0.815
Dcco-03	(CA) <sub>14</sub>	F-TACAATAAGCACACAGAATTGC R-GCGTTTGTCCCATTCTGA	54	224-226	2	0.208
Dcco-04	(AG) <sub>18</sub>	F-CATTCACTACATCCATCTGC R-TTGCCAGTCTCAGTCACA	54	241-279	15	0.742
Dcco-05	(AC) <sub>9</sub>	F-ATCAGTCTGATGCATCCAGC R-TGCTGATGACAGAGTCTTCAG	54	224-230	2	0.132
Dcco-06	(TG) <sub>14</sub>	F-CTGCATACTCTAAAGATGCC R-GCTTTTCAGTGACATTTTAAGTG	54	206-216	6	0.466

**Chapter 3. Phylogeography and population genetic structure of Double-crested Cormorants (*Phalacrocorax auritus*) inferred by mitochondrial control region sequences and microsatellite DNA**

**ABSTRACT**

We examined the genetic structure of Double-crested Cormorants (*Phalacrocorax auritus*) across their range in the United States and Canada to quantify variation within and among breeding sites and to assess the status of traditional geographically defined subspecies. Sequences (700bp) from domains I and II of the mitochondrial control region were analyzed for 234 Double-crested Cormorants from 23 breeding sites. Variation was also examined at eight microsatellite loci for 395 cormorants from the same 23 breeding sites. The mtDNA and microsatellite data provided strong evidence that the Alaska subspecies (*P. a. cincinnatus*) is genetically divergent from other populations in North America (net sequence divergence = 6.72%;  $\Phi_{ST}$  for mtDNA control region = 0.738;  $F_{ST}$  for microsatellite loci = 0.05). Our data also suggested strong genetic divergence in the southwestern U.S.; southern California may represent a zone of introgression resulting from a northward expansion of a unique lineage from the species range in northwestern Mexico. In contrast, there was little support for recognition of subspecies within the continental U.S. and Canada, outside of Alaska. Rather than genetically distinct regions corresponding to the putative subspecies [*P. a. albociliatus* (Pacific), *P. a. auritus* (Interior and North Atlantic), and *P. a. floridanus* (Southeast)], we observed a distribution of genetic variation consistent with a pattern of gradual isolation by distance.



This pattern implies that genetic differences across the range are due to geographic distance, rather than discrete subspecific breaks. Although three of the four traditional subspecies were not genetically distinct, potential demographic separation, habitat differences, and recent declines at some colonies within the regions, suggests that Pacific and possibly North Atlantic breeding regions may still warrant consideration as distinct populations.

## **INTRODUCTION**

The ability to describe population genetic structure and identify and order the responsible biological forces presents a continuing challenge. Genetic structure can be influenced by various forces, including gene flow, genetic drift, natural selection, and numerous ecological factors and life history traits. Recent and rapid advances in molecular methodologies and statistical analyses have aided this pursuit, providing for more rigorous examination of genetic structure and testing of theories in population genetics and phylogeography. Concurrently, interest in intraspecific population structure and delineation of conservation units has heightened as a result of increasing policy and legislation that allows for conservation and management of units below the species level (Haig et al. 2006). Information on the distribution of genetic variation within and among populations is necessary for the description of population structure and identification of conservation units to provide for the appropriate application of conservation and management efforts.

Avian taxa present an interesting case for identifying intra-specific groups as they tend to be highly vagile with large geographic distributions suggesting high gene flow, but may also exhibit strong natal philopatry and breeding site fidelity that restrict inter-population gene flow (Avice 2004). Phylogeographical patterns have been reconstructed mainly using maternally inherited mitochondrial DNA sequences (Avice 2000). For many avian species, the highly variable control region has proved useful for inferring intraspecific phylogenies (Wenink et al. 1996; Lucchini & Randi 1998; Abbott & Double 2003a; Haig et al. 2004; and others). Sole reliance upon mtDNA for identification of population structure and conservation units has been criticized, however, as patterns inferred from a single locus may be more reflective of the gene's idiosyncratic history than the species' history (e.g., Zink 2004, reviewed by Ballard and Whitlock 2004). More recently, development of hypervariable genomic markers, microsatellites, and new statistical methods have provided additional insights regarding evolutionary processes, demographic history, and population structure (Abbott & Double 2003b; Randi et al. 2003; Funk et al. 2007; Funk et al. 2008; and others). In this study, we used independent nuclear markers and mitochondrial DNA to describe the genetic structure of a controversial North American waterbird, the Double-crested Cormorant (*Phalacrocorax auritus*), across its range.

Double-crested Cormorants are colonial waterbirds with bicoastal and inland distributions in North America (Hatch and Weseloh 1999). They are habitat generalists occupying diverse aquatic environments including coastal waters, estuaries, lakes, rivers, ponds, and lagoons (Palmer 1962). Breeding colonies are located on rocky or sandy

islands, man-made structures such as bridges, docks, and transmission line towers, or in trees and shrubs near water (Chapdelaine and Bedard 1995; Stenzel et al. 1995; Hatch and Weseloh 1999). Colonies are highly variable in size, ranging from a few (rarely a single pair) to several hundred thousand pairs (Bent 1922; Palmer 1962). Their migratory nature is variable across the range; birds nesting in the interior and on the Atlantic coast are highly migratory and mostly winter in the Gulf of Mexico (Dolbeer 1991), while birds in other areas are primarily residential. Information about seasonal movement is limited, the degree of natal philopatry and breeding site fidelity has not been explicitly quantified, and any potential sex-based differences in these behaviors have not been characterized (Hatch and Weseloh 1999). Field studies suggest, however, that Double-crested Cormorants have fairly high breeding site fidelity and geographically limited dispersal (Dolbeer 1991; Hatch and Weseloh 1999; Clark et al. 2006). It is unknown whether dispersal is great enough to prevent substantial genetic divergence.

Double-crested Cormorants are commonly described as consisting of four subspecies occupying five more-or-less discrete geographic breeding regions; the nominate subspecies is comprised of two regional populations [Figure 3.1; American Ornithologists' Union (AOU) 1957; Palmer 1962; Bent 1992; Hatch 1995; Hatch and Weseloh 1999; Tyson et al. 1999; Wires and Cuthbert 2006]. The general geographic distributions (Figure 3.1) are as follows: *P. a. auritus* occupies the Interior and the North Atlantic regions, and has the widest subspecies distribution across northeastern and central North America; *P. a. floridanus* occupies the Southeast region, and breeds from Texas to Florida and north to the Carolinas; *P. a. cincinnatus* occupies the Alaska region,

and breeds solely in Alaska; *P. a. albociliatus* occupies the Pacific region, and breeds along the Pacific Coast from British Columbia south to Sinaloa, Mexico and inland, possibly to New Mexico, Utah, and Montana (Hatch 1995). More recently, a fifth subspecies (*P. a. heuretus*) was described by Watson et al. (1991) in the Bahamas. These subspecies were primarily based upon allopatry of the breeding regions. However, the geographic distributions are not entirely discrete, but overlap and are poorly defined in some locations (Hatch 1995). Furthermore, recent range expansion has obscured clear delineation of subspecific regions (Hatch and Weseloh 1999, Wires and Cuthbert 2006). For example, expansion into many previously unoccupied arid areas of the west has been facilitated by constructions of dams and reservoirs, Mid-Atlantic states have experienced local expansions, and development of aquaculture in the southeast has provided new habitat for colonies (Hatch and Weseloh 1999). Subspecies were also differentiated by size and crest character, but there is considerable variation in morphology, the distribution of crest characters is poorly known, and no significant phenotypic differences between subspecies have been characterized. In general, average size is described as increasing from south to north along both coasts and from east to west, and plumage varies across subspecific regions (Palmer 1962). Specifically, southeast birds are smallest and darkest with black crests, northeast birds have occasional white or partially-white crests, and north Pacific birds are largest and often have all-white crests (Palmer 1962).

Overall, Double-crested Cormorant numbers have increased dramatically since the 1970's, following extreme population declines primarily attributed to impacts of bioaccumulated pesticides (Hatch and Weseloh 1999). Although most populations

exhibited growth by the 1980's, the greatest growth rate and increase in number occurred within the Interior population, primarily in the vicinity of the Great Lakes (Hatch and Weseloh 1999). In contrast, the Alaska, North Atlantic, and parts of the Pacific populations exhibited negative rates of change from 1990-1994 (Tyson et al. 1999), with colony declines documented over much of southern Alaska, British Columbia, Washington, and southern California (Carter et al. 1995; Hatch and Weseloh 1999; Anderson et al. 2004). Growth of the North Atlantic population (Nova Scotia to Massachusetts) may have ceased by 1990 (Hatch and Weseloh 1999), the Pacific populations has yet to reach pre-pesticide numbers (Wires and Cuthbert 2006), and the Alaskan population is likely still experiencing declines (D.B. Irons, pers. comm.). A recent assessment of the Pacific subspecies, *P. a. albociliatus*, (USFWS unpubl. data) indicates a gradual continued increase in total population size; however, the distribution of the subspecies across its breeding range has changed dramatically and colony declines continue over much of British Columbia and parts of California (Moul and Gebauer 2002; Anderson et al. 2004; Capitolo et al. 2004; USFWS unpubl. data). Currently, nearly 50% of the Pacific population now nests in only a single colony located on East Sand Island, in the Columbia River estuary, Oregon (Anderson et al. 2004; Roby et al. 2008).

Recent counts estimate the total population to be 1-2 million individuals (about 350,000 breeding pairs), with large variation in population size among the subspecies (Hatch 1995). Numbers of breeding pairs in each subspecies were estimated as: *P. a. auritus* 257,000-357,000 pairs (Interior 170,000-270,000 pairs, North Atlantic 87,000 pairs); *P. a. floridanus* (Southeast) 9,400 pairs; *P. a. albociliatus* (Pacific) 33,000 pairs;

and *P. a. cincinnatus* (Alaska) 3,029 pairs (Wires and Cuthbert 2006). Additionally, the Bahamian population (*P. a. heuretus*) was estimated at only 212 pairs (Hatch 1995).

Recent changes in cormorant numbers and distribution have heightened human-cormorant conflicts and increased concern over possible impacts of high cormorant densities on other natural resources, particularly fisheries, resulting in differential and controversial management across the range. These concerns prompted the U.S. Fish and Wildlife Service (USFWS) and the U.S. Dept. of Agriculture/Wildlife Services (USDA/WS) to prepare an Environmental Impact Statement (USDI/FWS 2003a) and publish a final rule, which established a Public Resource Depredation Order for Double-crested Cormorants, effective in 24 eastern states. These rulings revised the 1998 Double-crested Cormorant Aquaculture Depredation Order to include lethal control at winter roost sites (USDI/FWS 2003b). Concurrently, large-scale management plans to reduce cormorant numbers have been implemented in several areas, including New England (Krohn 1995), the Great Lakes (Weseloh et al. 1995), and the St. Lawrence River (Bedard 1995). In contrast, Double-crested Cormorants in western states were not included in the Environmental Impact Statement or Depredation Order and are not actively managed for population control or conservation. Further, in British Columbia the species is designated as “threatened” on the provincial Red List due to declines in most colonies in the province (British Columbia Conservation Data Centre 2003).

An understanding of population structure and status is necessary to better inform conservation and management practices for the species across its range. Previous genetic analysis of the species is limited to two studies that exclusively examined the relationship

of southeastern U.S. cormorants relative to northeastern U.S. cormorants (Waits et al. 2003, Green et al. 2006). These studies failed to find significant genetic structure based on mitochondrial DNA sequence data (Waits et al. 2003) or microsatellite loci (Green et al. 2006), although both studies were limited by small samples sizes of populations and individuals.

The aim of this study was to examine and describe the genetic structure of Double-crested Cormorants in North America using a large number of breeding sites, individuals, mtDNA sequences, and microsatellite loci. Specific goals were to characterize the overall pattern of genetic differentiation within the species and identify and evaluate the genetic distinctiveness of individual breeding sites, *a priori* defined subspecies, or specific breeding regions.

## **METHODS**

### **Sample collection**

Blood or tissue samples were collected from 395 individuals at 23 breeding sites from throughout the range of the Double-crested Cormorant in North America (Table 3.1; Figure 3.1). Blood samples were collected following the protocol of the American Ornithologists' Union (Gaunt and Oring 1988). Tissue samples were obtained from heart, pectoral, or embryonic tissue from carcasses of individuals that had died of natural causes, were lethally collected under a depredation permit, or were collected for scientific research. Samples were stored in 2 ml cryogenic vials containing a buffer solution (100

mM Tris HCl, pH 8.0; 100 mM EDTA, pH 8.0; 10 mM NaCl; 0.5% SDS) and frozen at -80 °C until analysis. No known close relatives (parent/offspring, siblings, etc.) were included in the sampling. All samples were collected during the breeding season between 2002 and 2007. Additionally, a Brandt's Cormorant (*P. penicillatus*) and a Pelagic Cormorant (*P. pelagicus*) sample were obtained to serve as outgroups for phylogenetic analyses.

### **DNA extraction**

DNA was obtained by standard phenol/chloroform extraction. Briefly, 10 µl of blood or 1 mm<sup>3</sup> of tissue was digested in 400 µl of extraction buffer (50 mM Tris, pH 8.0; 10 mM EDTA, pH 8.0; 200 mM NaCl; 2% SDS) with Proteinase K (20 mg/ml) added to a final concentration of 600 µg/ml. Samples were vortexed and incubated overnight (~18 h) at 50 °C. If blood clots were not fully dispersed, a second aliquot of Proteinase K was added and the samples were incubated an additional 2 h. Samples were extracted with equal volumes of phenol (saturated with 10 mM Tris, pH 8.0) and then chloroform/isoamyl alcohol (25:1). DNA was cleaned and concentrated by centrifugation dialysis using Microcon 30,000 MW cutoff columns (Amicon Bioseparations). Stock DNA was diluted to a concentration of 100 ng/µl for experimental use.

### **Mitochondrial DNA methods**

An approximately 1.8 kb fragment containing the ND6 and control region of the mtDNA was initially obtained by long PCR using the primers L16087 (Desjardins and Morais



1990) and H1251 (Sorenson et al. 1999). Domain I and domain II of the control region were readily amplifiable and variable, while long repeats in domain III made sequencing and subsequent alignment problematic. Similar complex repeats have been reported in other seabirds (Abbott and Double 2003, Berg et al. 1995, Burg 2000). Forward primer DC01 (5'-TAGCCCTCAACCACAGGA-3') and reverse primer DC02 (5'-TTAGAAAGTTAGCGGTGGCG-3') were designed for specific amplification of a 900bp fragment containing sequence from domains I and II, located upstream of domain III. The final alignment contained 700 bps of the control region for all individuals examined. In addition, a 650 bp fragment of the cytochrome *b* gene was sequenced for a subset of 17 individuals using primers L14996 and H15646 (Sorenson et al. 1999). These data were used solely to confirm haplotype groups and estimate divergence times, and were not included in any other analyses.

Amplifications were performed using a PTC 100 thermal cycler (MJ Research). A total reaction volume of 20  $\mu$ l was used with the following concentrations: 10 mM Tris-HCl at pH 8.3; 50 mM KCl; 0.001% gelatin; 3.5 mM MgCl<sub>2</sub>; 100  $\mu$ M for each of the dNTPs; 0.2  $\mu$ M of each primer; 100 ng of template; and 1.5 U AmpliTaq Gold Polymerase (Perkin Elmer). The following parameters were used for amplifications: 12 min denaturation at 93 °C, followed by 35 cycles of 30 s at 93 °C, annealing at 50 °C for 30 s, and elongation at 72 °C for 1 min. A final 10 min elongation period at 72 °C followed the last cycle. PCR amplification quality was assessed by visualizing 5  $\mu$ l of the product with ethidium bromide on 1% agarose gels. Sequences were generated using ABI Prism Big Dye Terminator Cycle Sequencing chemistry on an ABI 3100 capillary DNA

automated sequencer (377 DNA Sequencer ABI Prism 377XL Collection Software) located in the Central Services Laboratory at Oregon State University. Ambiguities were resolved by comparing light and heavy-strand sequences or from overlap of different amplifications.

Use of avian blood as a DNA source could allow the amplification of nuclear homologs. However, our amplification of a large fragment as well as the use of bi-directional sequencing, increased the likelihood that sequences were mitochondrial in origin. Also, there was no relationship between tissue type and haplotypes, which would be expected if amplification of blood samples were resulting in nuclear homologs. Finally, there were no ambiguous peaks that occurred consistently across samples.

Alignment and manual adjustment of DNA sequences was completed using BIOEDIT version 7.0.5 software (Hall 2001). Alignment with the outgroup sequences, Pelagic Cormorant and Brandt's Cormorant, was performed using ClustalX (Thompson et al. 1997). Only unique haplotypes were included in matrices used for phylogenetic analyses. Phylogenetic inference was conducted with maximum parsimony criteria, maximum likelihood criteria, and Bayesian analysis. Parsimony analyses were conducted with program Paup\* 4.0b10 (Swofford 2000) using a heuristic search with 100 random addition-sequence replicates and tree-bisection-reconnection (TBR) branch swapping. Nodal support was assessed through nonparametric bootstrap analysis using 1,000 bootstrap replicates with 10 random addition-sequence replicates per bootstrap replicate. The most appropriate model of sequence evolution was selected using Akaike's information criteria (Akaike 1974) with the program Modeltest 3.7 (Posada and Crandall

1998). Likelihood analysis was then conducted in PAUP\* using successive iterations with starting parameters based on estimates from the previous tree (Sullivan et al. 2005). Parameters for the first tree were estimated from the most-parsimonious tree with the best likelihood score. Iterations were continued until successive searches yielded identical trees, likelihood scores, and model parameters. In addition, a log-likelihood ratio test was used to test the control region sequences for departure from a molecular clock, using the difference in likelihood from the best likelihood tree with and without a molecular clock enforced (Huelsenbeck and Crandall 1997).

Bayesian analyses were conducted in MRBAYES version 3.1.1 (Huelsenbeck and Ronquist 2003). Two replicate analyses, with four Markov chains each, were conducted simultaneously. Markov chains were run for seven million generations and sampled once every 100 generations. We used a conservative burn-in of 20,000 generations that was determined by examining stationarity of the likelihood scores and convergence of posterior probabilities between the two runs using the standard deviation of split frequencies. Additionally, we used the computer program NETWORK (available from [www.fluxus-engineering.com](http://www.fluxus-engineering.com)) to obtain a median-joining network of the analyzed haplotypes, except outgroup sequences (Bandelt et al. 1999). An initial star-contraction procedure (Forster et al. 2001), with a star connection limit of 2, was used to reduce the data set and provide a clearer presentation.

All 234 Double-crested Cormorant control region sequences were used for population genetic analyses and grouped as populations by breeding site (i.e., colony) and as *a priori* groups according to the four traditional geographic subspecies. Summary

statistics within and among sites were derived using ARLEQUIN version 3.01 (Excoffier et al. 2005). Genetic variation within sites was estimated with a variety of diversity statistics, including haplotype diversity ( $h$ ), number of polymorphic sites ( $s$ ), and nucleotide diversity ( $\pi_n$ ). Deviations from the assumptions of neutrality (Kimura 1983) were measured using Tajima's (1989)  $D$  statistic. Significant  $D$  values may be indicative of factors such as population bottlenecks or expansions. Genetic divergence among sites was estimated using  $F$ -statistics and analysis of molecular variance (AMOVA; Excoffier et al. 1992) in Arlequin version 3.01, which takes into account the number of mutations between haplotypes; a Tamura-Nei correction (Tamura and Nei 1993) was used for all calculations. Pairwise  $\Phi_{ST}$  values were calculated among all individual sites and for comparisons between subspecies. One thousand random permutations were used to test the probability of observing  $\Phi_{ST}$  values as large as or larger than those observed by chance, and the significance level for each test ( $\alpha$ ) was determined using a sequential Bonferroni adjustment (Rice 1989). For the AMOVA, we tested several *a priori* groupings of colonies to maximize  $F_{CT}$ , including grouping by site with no hierarchical structure and grouping by subspecies. One thousand random permutations were used to test the significance of variance components. We also calculated the corrected average percent sequence divergence, equivalent to net sequence divergence, between phylogenetically identified clades and subspecies in Arlequin.

### **Microsatellite methods**

Microsatellite primer sequences were obtained from two sources (Table 3.2). Initially, seven primer pairs isolated from the Great Cormorant (*P. carbo*) were tested for cross-species amplification (Piertney et al. 1998). From these, three loci (PcD2, PcT3, and PcT4) were polymorphic and gave reproducible results. Subsequently, microsatellite loci DCCO-01, DCCO-02, DCCO-03, DCCO-04, and DCCO-05 were obtained from microsatellite enriched clone libraries (Glenn and Schable 2005) developed specifically for this study (Chapter 2), and primer pairs were designed for the flanking sequence (Table 2.2).

For screening samples, PCR amplifications were performed in 10 µl reactions using the same conditions as described previously for mitochondrial amplifications. Annealing temperatures for primers are shown in Table 3.2. Amplification products were analyzed on an ABI 3100 capillary DNA automated sequencer located in the Central Services Laboratory at Oregon State University. ABI Genescan<sup>®</sup> analysis was used to determine fragment size based on internal lane standard GeneScan 500 [Rox]. ABI Genotyper<sup>®</sup> software was used to score alleles accurately. Of the 395 individuals scored at eight loci, only seven individuals did not amplify well for a single locus and subsequently were not scored for that locus.

MICROCHECKER software (Oosterhout et al. 2004) was used to check for the presence of null alleles and to assess the possibility of other genotyping errors, such as nonamplified alleles, short allele dominance, and scoring of stutter peaks. Linkage disequilibrium between loci across all sites and deviations from Hardy-Weinberg

genotype frequency for each locus and site were tested with GENEPOP version 3.4 (Raymond and Rousset 1995). Significance for these analyses was evaluated by Fisher exact tests, where  $P$  values were estimated by applying a Markov chain method. Sequential Bonferroni corrections were applied to determine the significance level of each test and correct for type I error (Rice 1989).

Genetic variation within sites was estimated with the diversity statistics: average gene diversity ( $H_E$ ), within population inbreeding coefficient ( $F_{IS}$ ), and allelic richness ( $R_S$ ), as calculated in FSTAT version 2.9.3.2 (Goudet 2001). Allelic richness is an index of the number of alleles corrected for sample size using rarefaction (El Mousadik and Petit 1996). Genetic divergence among sites was estimated with  $F$ -statistics and analysis of molecular variance using Arlequin version 3.01, as described above for mtDNA. A pattern of isolation by distance among individuals was tested using Alleles in Space (AIS; Miller 2005). This program implements a Mantel test using inter-individual genetic distances rather than genetic distances between sites, and thereby requires no *a priori* groupings of samples.

Population structure was further analyzed by performing a Principal Component Analysis (PCA) on the allelic frequencies for each breeding site. Data were arcsine square-root-transformed before analysis and used as input data for the PCA on the covariance matrix. Angular transformation was chosen due to its applicability to percentage and proportion data (Sokal & Rohlf 1995, p.419). Analyses were performed with S-Plus version 7.0.2. We then plotted PC2 versus PC1 and PC3 versus PC2 to

estimate genetic divergence as the relative linear distance between points representing each breeding site.

We also used a Bayesian clustering approach implemented in program STRUCTURE version 2.2.3 (Pritchard et al. 2000) to estimate the number of populations ( $K$ ) and assign individuals to one or more of these populations ( $k$ ). This approach groups populations by minimizing deviations from Hardy-Weinberg equilibrium and linkage equilibrium between loci within populations. We used the admixture model that assumes gene flow among populations and correlated allele frequencies. This model assigns a proportion of each individual's genome to each population ( $q_k$ ). STRUCTURE was run for  $K = 1$  to  $K = 10$  clusters. Each run was pursued for 1 million Markov chain Monte Carlo (MCMC) iterations, with an initial burn-in of 50,000, which gave consistent results in preliminary runs. Five independent simulations were run for each  $K$  to assess stability, and the mean  $\ln P(d)$  across runs for each  $K$  was calculated. The final posterior probability of  $K$  was computed according to Pritchard et al. (2000), using the runs with the highest probability for each  $K$ . An additional ad hoc statistic ( $\Delta K$ ) was also estimated, as it was shown to be a better predictor of the true  $K$  at the uppermost hierarchical level (Evanno et al. 2005).

## RESULTS

### Mitochondrial DNA variation

Mitochondrial sequences containing 700 base pairs (bp) of domain I and domain II of the control region were obtained for 234 Double-crested Cormorants sampled from 23 breeding sites. In total, 87 unique haplotypes were detected. In the final alignment, 116 of 700 total characters were variable, of which 104 were parsimony informative. There were no insertions or deletions. With the inclusion of two outgroup sequences, one Brandt's Cormorant and one Pelagic Cormorant, the alignment required four indels, resulting in a final sequence length of 704 bp. The high percentage of haplotypes to individuals sequenced (ca. 37%) and the rapid substitution rate observed for the fragment argued against the likelihood of having sequenced a slower evolving nuclear copy (Sorenson & Fleischer 1996).

Genetic diversity in the sampled cormorants was substantial. Only one haplotype (haplotype 11) was common and geographically widespread, occurring in 27 individuals sampled in the Pacific, Interior/North Atlantic, and Southeast subspecies. The other common haplotype (67) was the most common Interior/North Atlantic haplotype and was found in 10 individuals from this subspecies. All other haplotypes were found in less than 10 individuals, with most occurring only once (45%) or twice (27%). Consequently, there were many haplotypes restricted to a single site and thereby subspecies. Among the 87 total haplotypes found, unique haplotypes included: 6 in Alaska; 24 in the Pacific; 32 in



the Interior/North Atlantic; and 9 in the Southeast. Finally, there were 16 haplotypes shared among subspecies, although Alaska shared no haplotypes with other sites.

Within population genetic diversity was generally high, although haplotype and nucleotide diversity exhibited a range of values across sites (Table 3.1). Haplotype diversity was lowest in Kenai, Alaska (0.66) and in the Aleutians, Alaska (0.70), and highest in Alberta, Minnesota, Wisconsin, and Imperial, California (1.00). Nucleotide diversity was also lowest in Kenai, Alaska (0.0067) and in the Aleutians, Alaska (0.0063), and highest in Imperial, California (0.0533) and Grays Harbor, Washington (0.0553). At no site did Tajima's  $D$  statistic differ significantly from expectations under neutrality, providing no evidence of population bottlenecks or expansion. Overall  $D$  for cormorants was 0.50 ( $P > 0.10$ ).

Phylogenetic analyses of the aligned control region sequences of unique haplotypes indicated similar topologies for all methods of tree inference (Figure 3.2). Maximum-parsimony analysis generated 6,084 most parsimonious trees of 367 steps (CI = 0.624, RI = 0.900). The best model of sequence evolution chosen by Modeltest 3.7 was a GTR+I+G model (general time reversible model with variable base frequencies and six substitution rates, a proportion of invariant sites, and among-site rate heterogeneity). The maximum likelihood analysis only required two iterations to reach convergence of tree topologies and branch lengths. The negative log-likelihood score of the final tree was 2,824.92 (estimated base frequencies: A: 0.2875, C: 0.1938, G: 0.1733, T: 0.3453; rate matrix: A-C: 2.309, A-G: 36.050, A-T: 1.100 e-10, C-G: 1.048, C-T: 30.882, G-T:

1.000; shape parameter for gamma distribution: 0.4144; proportion of invariant sites: 0.4885).

In the Bayesian analysis, plots of model parameters and likelihood scores indicated that stationarity was reached by generation 100,000. Further, the standard deviation of split frequencies between runs indicated that convergence had also been achieved after removal of a conservative burn-in of 20,000 generations. Thus, the last 50,000 sampled trees in each of two runs were combined to yield 100,000 trees for the final Bayesian posterior probabilities (bpp). The 50% majority rule consensus tree of all sampled Bayesian trees had 41 nodes with greater than 50% support, 38 of which were also observed in the maximum likelihood tree. Bayesian posterior probabilities for these nodes are shown on the maximum likelihood tree (Figure 3.2).

Many nodes were well-supported in all analyses (parsimony, likelihood, and Bayesian), although only a few clades showed strong association with geography and no subspecies was monophyletic (Figure 3.2). However, all analyses yielded nearly identical tree topologies with a basal structure of three major clades having geographic affiliation. All Alaskan haplotypes (haplotypes 1, 2, 3, 4, 5, and 6) along with two Pacific haplotypes (9 and 18) formed one distinct clade (hereafter termed the “northwestern lineage”). A second distinct clade (hereafter termed the “southwestern lineage”) consisted of haplotypes 8, 25, 27, 28, 29, and 32, corresponding to 12 individuals from the Pacific, seven of which were sampled in southern California. Between these two lineages were four intermediary haplotypes (19, 20, 26, and 39), corresponding to four individuals sampled within the Pacific. The third and largest clade (hereafter termed the “continental

clade”) contained the remaining 69 haplotypes, and included individuals from all of the subspecies, except for Alaska. Within this large clade, there were many well-supported sub-clades although there was only shallow divergence and little geographic structure.

The median-joining network, generated in Network, was congruent with the phylogenetic analysis and consisted of three primary clades, as well as an intermediary group of four haplotypes, separated by numerous mutational steps (Figure 3.3).

Constitution of these groups was identical to the phylogenetic clades described above. Additionally, the network illustrated that within the main clade many haplotypes were shared between subspecies and all haplotypes were highly similar, with less than five mutational steps between any adjacent nodes.

The log-likelihood ratio test (lrt) of the best-fit tree, with and without a molecular clock enforced, rejected the clock-like tree at a 5% significance level (2824.92 versus 2244.53,  $lrt = 580.39$ ,  $P < 0.001$ ,  $df = 85$ ), suggesting the Double-crested Cormorant control region was not evolving in a clock-like manner. Further, outgroup sequences were evidently too distantly related to provide a reliable root, and placement of the root varied by phylogenetic method. Cytochrome *b* sequence was obtained for 17 individuals representing the major phylogenetic groups including: four individuals from the northwestern lineage, three from the intermediate group, three from the southwestern lineage, and seven from the continental clade. There were nine fixed differences observed between the two western lineages and the continental clade (corrected average percent sequence divergence: 1.48%), two fixed differences between the southwestern lineage and the northwestern lineage (corrected average percent sequence divergence: 0.31%),

and no differences between the northwestern lineage and the intermediate group.

Assuming the avian cytochrome *b* gene evolves in a clock-like manner at a rate of 2% per million years (Weir and Schluter 2008), the initial divergence between the continental clade and the western lineages could be estimated at approximately 740,000 years ago, and the divergence between the northwestern and southwestern lineage occurred more recently, approximately 154,000 years ago.

Corrected average percent sequence divergence (equivalent to net percent sequence divergence) between different groupings of cormorants is shown in Table 3.3. The greatest net sequence divergence was observed in comparisons between Alaska and the other subspecies, with 6.72% ( $P < 0.001$ ) divergence between Alaska and all other subspecies. Outside of Alaska, net sequence divergence between subspecies was low but statistically significant for comparisons between the Pacific and Interior/North Atlantic, and between the Pacific and Southeast, but not significant for comparison between the Interior/North Atlantic and Southeast. Overall, the Pacific and North Atlantic cormorants were more divergent (0.52% and 0.44% sequence divergence, respectively) than the Interior or Southeast cormorants (0.12% and 0.10%, respectively). Finally, sequence divergence between phylogenetically identified clades was greatest, with 8.0% divergence between the western lineages and continental clade, and 4.0% divergence between the northwestern and southwestern lineages.

Population genetic analyses also revealed a non-random distribution of genetic variation among sites that was consistent with the phylogenetic analysis (Table 3.4). The overall  $\Phi_{ST}$  value among all sites was large (0.476) and statistically significant

( $P < 0.001$ ). Pairwise  $\Phi_{ST}$  values ranged from negative values up to 0.927 between Kenai, Alaska and Maryland. Of the 253 pairwise comparisons, 150 comparisons were significant at  $\alpha = 0.05$  level, and 82 comparisons were significant after sequential Bonferroni correction. The matrix of pairwise  $\Phi_{ST}$  values revealed that all significant comparisons involved a distally located site, and nearly all involved an Alaskan site (Kenai and Aleutians, Alaska) or a North Atlantic site (Nova Scotia, Maryland, Massachusetts, and Pennsylvania). Thirty-nine pairwise comparisons (93%) between Alaska and other sites were significant, with a mean  $\Phi_{ST}$  of 0.822 (ranging from 0.378 to 0.929). Forty-four pairwise comparisons (58%) between the North Atlantic and other sites were significant, with a mean  $\Phi_{ST}$  of 0.348 (ranging from 0.105 to 0.929). The remaining five significant comparisons were between Pacific sites and South Carolina. Additionally, large  $\Phi_{ST}$  values were observed in comparisons between San Diego, California and other sites (mean: 0.497; range: -0.001 - 0.685), but most values were not significant after Bonferroni correction.

Considering *a priori* groupings by subspecies, the greatest pairwise  $\Phi_{ST}$  value was observed between Alaska and all other subspecies ( $\Phi_{ST} = 0.738$ ). Outside of Alaska, comparisons between the Pacific and other subspecies ( $\Phi_{ST} = 0.186$ ) and the Interior/North Atlantic and other subspecies ( $\Phi_{ST} = 0.067$ ) were slight but significant, while comparison between the Southeast and other subspecies ( $\Phi_{ST} = 0.029$ ) was not significant. Furthermore, within the eastern range, comparison between the North Atlantic and the Interior regions ( $\Phi_{ST} = 0.209$ ) was significant.

*A priori* groupings of Double-crested Cormorants were supported by AMOVA, with all tests yielding significant variance components (Table 3.5). A single-level AMOVA indicated large and significant differentiation among sites, as well as high variation within sites (47.62% variation among sites,  $P < 0.001$ ; 52.38% variation within sites,  $P < 0.001$ ). Significant variation was also found among sites within the major geographic regions defined by subspecies. In the Pacific, among site differences explained 12.53% of the variance ( $P = 0.003$ ). In the east, including the Interior/Atlantic and Southeast, among site differences explained 13.13% of the variance ( $P < 0.001$ ). A grouping of sites by the four subspecies was statistically significant and explained 48.09% of the variation as among group variation ( $P < 0.001$ ), while 44.95% of the variation was attributed to within site variation. However, this *a priori* grouping produced similar variance components as the analysis with no hierarchical grouping at all. In contrast, grouping the three most distal locations of Alaska, the North Atlantic, and San Diego, California independently and separate from the rest of the combined sites could explain 58.05% of the variation as among group variance. Yet, a further simplified grouping of three, with Alaska and San Diego, California separated from the rest of the combined sites, could best maximize the among group variance component while minimizing the among sites and within sites variance components (71.68% variation among groups,  $P < 0.001$ ; 4.74% variation among sites,  $P < 0.001$ ; 23.58% variation within sites,  $P < 0.001$ ).

**Microsatellite variation**

Across the full data set, genotypic frequencies conformed to expected Hardy-Weinberg proportions for all eight loci and there was no evidence of linkage disequilibrium between any pairs of loci. No loci showed consistent evidence of null alleles across sites, and MICROCHECKER detected no other potential scoring errors. Of 184 tests for deviation from Hardy-Weinberg proportions, only 11 were significant at  $\alpha = 0.05$  level. This was slightly more than the 9.2 tests expected to deviate by chance. After sequential Bonferroni corrections were applied to tests for each locus, departures from Hardy-Weinberg equilibrium were found in only two cases: Kenai, Alaska and Grays Harbor, Washington for locus PcT4. Of the 644 tests for linkage disequilibrium, only 18 tests were significant at  $\alpha = 0.05$  level, which was less than would be expected by chance. Further, no pairs of loci showed consistent deviations across populations. Only three tests were found to be significant after sequential Bonferroni corrections were applied to tests for each pair of loci: loci DCCO-02 and PcT4, loci DCCO-01 and PcD2, and loci PcT3 and PcD2 in Grays Harbor, Washington.

Overall the eight microsatellite loci analyzed yielded similarly high levels of genetic diversity for all sampled sites. The average allelic richness per site was 4.70, ranging from 3.87 (Kenai, Alaska) to 5.23 (Imperial, California), and expected heterozygosity averaged across loci ranged from 0.593 (Kenai, Alaska) to 0.732 (Maryland). Allelic fixation occurred at locus DCCO-03 and locus DCCO-05, which each had only two alleles sampled. For locus DCCO-03, the Kenai, Alaska; Aleutians, Alaska; Grays Harbor, Washington; San Diego, California; and Idaho sites were all fixed

for allele 1. For locus DCCO-05, the Aleutians, Alaska; San Diego, California; Massachusetts; Pennsylvania; and South Carolina sites were all fixed for allele 2. A total of sixteen unique alleles were sampled. The Quebec, Nova Scotia, Massachusetts, Maryland, and Pennsylvania site each had one unique allele. The Saskatchewan and the Walla Walla, Washington site each had two unique alleles at two loci. Finally, Alaska and San Diego, California had the highest frequency of unique alleles; Kenai, Alaska had three unique alleles at two loci, the Aleutians, Alaska had one unique allele, and San Diego, California had three unique alleles at one locus. All unique alleles occurred at low frequencies less than 0.1, except for allele 11 and allele 13 at locus PcT4 in San Diego, California, which occurred at high frequencies of 0.25 and 0.33.

The overall  $F_{ST}$  value among all sites was 0.029 and overall genetic differentiation was statistically significant ( $P < 0.001$ ). Pairwise  $F_{ST}$  values ranged from negative values up to 0.149 between the Aleutians, Alaska and San Diego, California. Of the 253 pairwise comparisons, 175 comparisons were significant at  $\alpha = 0.05$  level and 104 comparisons were significant after sequential Bonferroni correction. The matrix of pairwise  $F_{ST}$  values among all sites (Table 3.6) revealed that all significant comparisons involved a distally located site. In agreement with the mitochondrial  $\Phi_{ST}$  values, nearly all significant comparisons involved either an Alaskan site (Kenai and Aleutians, Alaska) or a North Atlantic site (Nova Scotia, Maryland, Massachusetts, and Pennsylvania). Thirty-four pairwise comparisons (81%) between Alaska and other sites were significant, with a mean  $F_{ST} = 0.075$  (ranging from 0.045 to 0.149). Thirty-eight pairwise comparisons (50%) between the North Atlantic and other sites were significant, with a



mean  $F_{ST} = 0.038$  (ranging from 0.004 to 0.106). Similar to mitochondrial results, large  $F_{ST}$  values were also observed in comparisons between San Diego, California and other sites (mean: 0.064; range: 0.032-0.149), and Grays Harbor, Washington and other sites (mean: 0.061; range: 0.045-0.105). Unlike with the mitochondrial data, several of these comparisons were significant: 12 comparisons (55%) involving San Diego, California and 20 comparisons (91%) involving Grays Harbor, Washington were significant. Finally, the remaining 15 significant values involved comparisons between the Pacific and sites located in the eastern United States.

Considering groupings by subspecies, the largest significant pairwise  $F_{ST}$  value was observed between Alaska and all other subspecies ( $F_{ST} = 0.053$ ). Outside of Alaska, comparisons between the Pacific and other subspecies ( $F_{ST} = 0.014$ ) and the Interior/North Atlantic and other subspecies ( $F_{ST} = 0.003$ ) were small but significant, while a comparison between the Southeast and other subspecies ( $F_{ST} = 0.004$ ) was not significant. Furthermore, within the eastern range, comparison between the North Atlantic and the Interior regions ( $F_{ST} = 0.016$ ) was small but significant.

*A priori* groupings of Double-crested Cormorants were supported by AMOVA (Table 3.5). Although significant variation among groups could be found for many *a priori* groupings, the majority (greater than 90%) of the total variation was attributed to variation among individuals within sites for all comparisons. A single-level AMOVA indicated small but significant variation among sites and high variation within sites (2.84% variation among sites,  $P < 0.001$ ; 97.16% variation within sites,  $P < 0.001$ ).

Significant variation was also found among sites within the major geographic regions defined by subspecies. In the Pacific, among site differences explained 2.25% of the variance ( $P < 0.001$ ). In the east, including the Interior/North Atlantic and the Southeast, among site differences explained 1.47% of the variance ( $P < 0.001$ ). A grouping of sites by the four described subspecies was statistically significant, but could only explain 1.64% of the variation as among group variation ( $P < 0.001$ ), while 96.66% of the variation was attributed to within site variation. As with the mitochondrial data, grouping the three most distal locations of Alaska, the North Atlantic, and San Diego, California independently from the rest of the combined sites could explain more of the among group variance than the *a priori* grouping by subspecies (2.71% variance among groups,  $P < 0.001$ ; 1.68% variance among sites,  $P < 0.001$ ; 95.61% variance within sites,  $P < 0.001$ ). Yet, the further simplified grouping of three, with Alaska and San Diego, California grouped independently from the rest of the combined sites, maximized the among group variance component while minimizing the among sites and within sites variance components (4.41% variation among groups,  $P < 0.001$ ; 2.05% variation among sites,  $P < 0.001$ ; 93.54% variation within sites,  $P < 0.001$ ).

The principal component analysis performed on allelic frequencies per site did not identify distinct clusters of sites by subspecies or otherwise (Figure 3.4). Rather, the coordinate axes suggested a relationship between genetic and geographic distance, consistent with a pattern of isolation by distance. The total genetic variance explained by the first, second, and third principal components was 21.3%, 12.7%, and 9.2%, respectively. The first principal component axis (PC1) placed all sites in a linear order

that was nearly identical to their geographic order from west to east. The Alaskan sites had the lowest PC1 scores, while the North Atlantic sites had the highest PC1 scores. Along this axis, the two Alaskan sites were well separated from all other sites; the Interior and Southeast sites were all closely associated with very similar PC1 scores; and the Pacific sites were more separated from each other than were sites within other regions. The alleles with the most influence in this discrimination, as indicated by the coefficients of the first eigenvector, were all common alleles that showed a clear longitudinal gradient in allele frequency (allele 2/locus DCCO-03; allele 2/locus DCCO-02; and alleles 4, 6, and 14/PcD2). The second principal component axis (PC2) highly separated San Diego, California from all other sites, identified the Aleutians, Alaska and Modoc, California as slightly differentiated, and showed little differentiation amongst the other sites. The alleles with the greatest influence on PC2, identified by the coefficients of the second eigenvector, included two alleles that were common but not present in the Alaskan sites (allele 7/locus DCCO-02 and allele 4/locus DCCO-04), one allele that was common overall but not present in San Diego, California (allele 8/locus DCCO-01), and three alleles that were unique to San Diego, California (alleles 9, 11, and 13/locus PcT4). The third principal component (PC3) did not identify any one site as highly differentiated, but did illustrate a similarity amongst distal sites versus other sites. Specifically, the most distal sampling sites of the Aleutians, Alaska; San Diego, California; Massachusetts; and Nova Scotia had the highest PC3 scores. Other distal sites, including Kenai, Alaska; Grays Harbor, Washington; Pennsylvania; Maryland; and South Carolina had moderate PC3 scores. The remaining Interior, Southeast, and Pacific sites

had low PC3 scores. The alleles with the most influence for PC3, identified by the coefficients of the third eigenvector, were alleles that had been lost from the distal sites of the Aleutians, Alaska and San Diego, California and also had low or zero frequency in the other distal sites (allele 1/locus DCCO-05; allele 1/locus DCCO-02; allele 8/locus DCCO-04; and allele 7/locus DCCO-02).

In the STRUCTURE analysis, the number of clusters ( $K$ ) with the highest mean log probability of the data [ $\ln P(D) = -11333.4$ ] and a posterior probability close to 1.0 was  $K = 2$  (Figure 3.5(a)). However, the mean  $\ln P(D)$  was only 21.7 lower for  $K = 3$  and  $\Delta K$  was higher for  $K = 3$  than  $K = 2$  ( $\Delta K = 74.16$  and  $18.94$ , respectively) (Figure 3.5(b)). Therefore, we chose  $K = 3$  as the most appropriate number of cormorant population clusters following the recommendation of Evanno et al. (2005), who found that in most cases the ‘log probability of the data’ did not provide a correct estimation of the number of clusters while the ad hoc statistic  $\Delta K$  did accurately detect the number of clusters.

The STRUCTURE analysis did not support grouping of cormorants by subspecies or identify discrete clusters (Figure 3.6). However, the three clusters identified did have a relationship with geography and mean membership of sites in each cluster was clinal. The Alaskan sites had the highest mean membership in a single cluster, with a mean membership of 73.9% in cluster 3. Similarly, the North Atlantic sites had the highest mean membership in cluster 1, with a mean membership of 61.8% in the cluster. Finally, the San Diego, California site was the only site with a large mean membership for cluster 2, with a mean membership of 66.0% in the cluster. The STRUCTURE analysis also indicated clinal variation as illustrated by the mean membership of sites in cluster 1

versus cluster 3. Specifically, sites in the Pacific proximal to Alaska had greater membership in cluster 3 (range: 35.7% to 69.4%), than sites outside of the Pacific (range: 11.9% to 26.9%). Concordantly, mean membership in cluster 1 was greater in Interior and Southeast sites proximal to the North Atlantic sites (range: 29.2% to 51.1%), than in Pacific and Alaskan sites (range: 6.4% to 29.2%). Thus, mean membership in cluster 3 showed a pattern of decreasing from west to east while mean membership in cluster 1 showed an inverse pattern of decreasing from east to west. Additionally, mean membership in cluster 2 for sites outside of San Diego, California ranged from 16.0% for Kenai, Alaska to 48.0% for Imperial, California with some tendency for mean membership to increase with geographic proximity to San Diego.

Mantel tests suggested a significant relationship between geographic distance and genetic distance between individuals when all individuals were compared ( $r = 0.092$ ,  $P = 0.001$ ). Additionally, there was a slightly stronger relationship when just Pacific individuals were compared ( $r = 0.086$ ,  $P = 0.001$ ) than when individuals within the Interior, North Atlantic, and Southeast were compared ( $r = 0.061$ ,  $P = 0.002$ ).

## **DISCUSSION**

Results from this study of Double-crested Cormorants in North America provided a thorough characterization of genetic variation and differentiation within the species. The strongest result was the presence of phylogenetic structure that identified two primary phylogeographic breaks. Population genetic analyses of mitochondrial and microsatellite

data were concordant and supported the phylogenetic structure detected. The combined microsatellite and mitochondrial data provided strong evidence that Double-crested Cormorants from Alaska are genetically distinct from other populations and strongly suggested that Double-crested Cormorants from the southwestern portion of the range may also be unique, but provided little evidence of discrete genetic breaks between subspecies within the rest of the continental U.S. and Canada.

### **Phylogeographic structure**

Phylogeographic analysis of mtDNA data identified a few main clades that were highly supported and divergent. The phylogenetic analysis and median-joining network split the mtDNA phylogeny into three primary genetic subsets: a deep partition between a large continental clade and a western group, and a subsequent partition of the western group into a northwestern lineage and a southwestern lineage (Figure 3.2 and 3.3). The northwestern lineage had the strongest geographic identity, being primarily restricted to Alaska and containing all haplotypes sampled within Alaska. The southwestern lineage also had a clear geographic association as it primarily contained individuals sampled in southern California, including four of the six San Diego, California cormorants and three of the 10 Imperial, California cormorants. In contrast, the continental clade was characterized by shallow divergence between haplotypes and a general lack of association of subclades with geography. The only noticeable relationship between phylogenetic and geographic structure was the concentration of all haplotypes sampled in the North Atlantic to one sub-clade. However, many of these haplotypes were not unique

to the North Atlantic and the sub-clade also contained individuals and haplotypes from other regions.

These three described clades were separated by long branches with the only intermediaries being a less-supported group of four haplotypes from the Pacific, occurring between the northwestern and southwestern lineages. This structure implies past fragmentation associated with historical vicariance events producing the observed genetic discontinuities (Avice 2000). The deep divergences observed may be explained by Late Pleistocene events that have been postulated as driving speciation in many species (Hewitt 1996), as well as substantial microevolutionary genetic diversification in birds (Avice and Walker 1998). An alternative hypothesis is that deep phylogeographic breaks can occur in a continuously distributed species if individual dispersal distances and/or population sizes are low (Irwin 2002). This would result from low dispersal, high philopatry, and the mode of mtDNA inheritance. This hypothesis could be true for the western lineages of Double-crested Cormorant given their demography and residential nature (Hatch and Weseloh 1999), and would not exclude elements of the Pleistocene hypothesis introduced above.

### **Population genetic structure**

The combined analyses of the eight microsatellite loci corroborated the major findings of the mitochondrial data. Population genetic analyses of both markers were congruent and supported the inferred phylogenetic structure. Specifically, both data sets identified the Alaskan sites and the San Diego, California site as the most genetically distinct areas

within the Double-crested Cormorant, and supported differentiation of the North Atlantic sites. The pairwise genetic distance matrices for the two markers were highly correlated (Mantel test for correlation:  $r = 0.76$ ;  $P = 0.0010$ ) and revealed significant differences in mitochondrial and nuclear allele frequencies for comparisons involving these distal sites. Likewise, AMOVA analysis revealed that the Alaskan and San Diego, California sites were responsible for most of the among group variance present within the data, as these two locations contained the most divergent and unique haplotypes and genotypes. Further, it should be noted that Alaska and San Diego, California contained unique microsatellite alleles and mitochondrial haplotypes. The presence of unique alleles at multiple independent loci supports the conclusion that unique cormorant lineages are present in these areas, rather than just a unique gene lineage as could result from idiosyncrasies of the lineage sorting process.

## **Regional Structure**

### *Northwestern*

The unique lineage observed in Alaska may be explained by a process of post-glacial recolonization. The pattern of decreased genetic diversity and differentiation observed in Alaska is congruent with a relatively recent origin from a subset of the original gene pool (Hewitt 1996). The serial bottlenecks associated with extirpation and recolonization, as can result from glaciation events, could produce such decreases (Hewitt 2000). Specifically, mitochondrial diversity was notably low within Alaska and higher levels of nuclear genetic diversity can be explained by these markers' typically high mutation rate



that allows for faster recovery of variation after a reduction in population size (Nei et al. 1975; Lande and Barrowclough 1987). The clade was not monophyletic for Alaska, however, and contained two Pacific haplotypes. Lack of complete phylogenetic resolution is expected, as retention of ancestral polymorphism leading to incomplete lineage sorting would be likely given the relatively recent divergence and overall large population size (Avice et al. 1984, Avice 1989). Although, the presence of haplotypes unique to the lineage in the Pacific could simply represent recent long-distance dispersal of Alaskan cormorants southward.

Phylogeographic and genetic data indicate that, except for strict monophyly, Alaskan cormorants meet the common genetic criteria for avian subspecific status (Funk et al. 2007) and recognition as an evolutionarily significant unit (Moritz 1994a, b), including significant variation in microsatellite allele and mtDNA haplotype frequencies, the presence of unique alleles at multiple loci, and significant net sequence divergence. First, all pairwise  $\Phi_{ST}$  values for mtDNA haplotypes between Alaska and other sites were large, and most were statistically significant. Further, the pairwise  $\Phi_{ST}$  value between Alaska and other subspecies (0.738) was within the range of values observed among avian subspecies at the mtDNA control region (0.036-0.950; Fry and Zink 1998; Valliantoes et al. 2002; Benedict et al. 2003; Eggert et al. 2004; Pitra et al. 2004; Solorzano et al. 2004). Second, all six Alaskan haplotypes were unique to the subspecies and formed a well-supported phylogenetic clade. Third, net sequence divergence between Alaskan individuals and all others was substantial (6.72%), statistically significant, and well within than the range of values observed among recognized avian subspecies for the

mtDNA control region (0.49 – 8.0%; Quinn 1992; Edwards 1993; Fry and Zink 1998; Bhagabati et al. 2004). Finally, the pairwise  $F_{ST}$  value between Alaska and other populations (0.055) was within the range of values observed among recognized avian subspecies at microsatellite loci (0.023-0.571; Chan and Arcese 2002; Eggert et al. 2004; Pitra et al. 2004; Jones et al. 2005).

Historical records, contemporary population estimates, and field observations are consistent with recognition of Alaskan Double-crested Cormorants as distinct and potentially of conservation interest. The Alaskan population is the most geographically isolated of the subspecies owing to a disjunct distribution along the Pacific Coast of North America, with no nesting colonies occurring along the coast of British Columbia north of the Strait of Georgia (Carter et al. 1995). Further, Alaskan and Pacific birds are mostly resident within their breeding range and seasonal movement of Alaskan birds may only extend to northern British Columbia (Hatch and Weseloh 1999). This geographic separation likely limits movement between regions and suggests that the Alaskan subspecies is also demographically distinct. In addition, the Alaskan subspecies suffered population declines and range reduction starting in 1750 and continuing into, at least, the 1900's primarily owing to introduction of foxes onto islands and human disturbance (Carter et al. 1995). The Alaskan subspecies currently has the smallest population size of the purported subspecies examined (3,029 breeding pairs), consisting of only 126 colonies, most being less than 100 pairs (Wires and Cuthbert 2006). This contemporary population is less numerous and less widespread than historically (Wires and Cuthbert

2006) and may still be experiencing declines (Carter et al. 1995; Tyson et al. 1999; DB Irons, pers. comm.).

### *Southwestern*

In contrast to Alaska, the San Diego and Imperial, California sites had high genetic diversity, particularly nucleotide diversity. This result is best explained as a consequence of the confluence of lineages of multiple origins at these sites (Petit et al. 2003). In particular, of 16 individuals analyzed from this area, seven contained haplotypes found in the southwestern lineage, while the remaining nine were allied with sequences from the continental clade. From this pattern, it is evident that the genetic differentiation of the area is not due to a strict genetic discontinuity or overt geographical barrier. Rather the presence of unique haplotypes at these sites likely reflects introgression of southern alleles into more northern regions. Additionally, two haplotypes unique to this lineage were also found in cormorants from Oregon and Washington. Thus, southern California may represent the primary zone of introgression, while the extent of migration may be as far north as Washington, or further.

A northward expansion of a southern lineage is consistent with field observations and historical records. The southern limit of the breeding range of the Double-crested Cormorant extends to Baja, California Sur and Sinaloa, Mexico, although our sampling was limited to California. The largest cormorant colony ever recorded on the continent existed at San Martin Island, Baja California, MX, and has been estimated as 213,500 pairs (Hatch 1995). This colony disappeared entirely in the late 1970's, with no nests

found in 1977, primarily owing to human disturbance and introduced predators (Gress et al. 1973, Everett and Anderson 1991). Additional declines in colonies and productivity were documented in Mexico, including the near extirpation of 2,000 breeding birds from Isla Los Coronados, during the early to late 20<sup>th</sup> century (Gress et al. 1973, 1995). It was suspected that many of these birds moved northward to nest at other colonies (Carter et al. 1995). Concurrently, numbers of breeding cormorants in southern California increased strikingly from 416 birds in 1975-77 to 2,528 birds in 1991, including dramatic increases at the Salton Sea. This rate of increase could not be explained by local productivity alone and has been attributed to immigration of birds from Mexico (Carter et al. 1995; Wires and Cuthbert 2006). Thus, it is likely the unique haplotypes observed in southern California may be more allied with Mexican populations than with cormorants from the continental U.S., and thereby represent northward movement and introgression between previously isolated lineages. Additional sampling will be necessary, however, to describe the identity of this lineage. As the geographic extent of haplotypes and genotypes and the uniqueness of the suspected lineage is further defined and examined, the southern range may warrant consideration as a distinct population, subspecies, or conservation unit.

### *Continental*

There was little genetic evidence for subspecies level differentiation of cormorants within the conterminous U.S. and Canada, outside of Alaska. First, there were no mtDNA clades restricted to sites in the western or eastern portion of the species' range corresponding to subspecies. Second, the pairwise  $\Phi_{ST}$  values between the Pacific and other subspecies

(0.186) and the Interior/North Atlantic and other subspecies were at the low range of values reported for other avian subspecies at the mitochondrial control region (see citations above), while the pairwise  $\Phi_{ST}$  value between the Southeast and other subspecies (0.029) was below the range. Third, pairwise  $F_{ST}$  values between subspecies (Pacific versus others: 0.014; Interior/North Atlantic versus others: 0.003; Southeast versus others: 0.004) were below the range of values reported for other recognized avian subspecies at microsatellite loci (see citations above). Fourth, grouping sites by subspecies provided no better explanation for the partitioning of genetic variation than no hierarchical grouping at all in the AMOVA analyses. Further, the multivariate analysis (PCA) and Bayesian clustering analysis (STRUCTURE) did not identify distinct clusters of sites by subspecies. The shallow sequence divergence and lack of phylogenetic resolution observed within the continental clade coupled with the low  $\Phi_{ST}$  and  $F_{ST}$  values indicate that sites have been connected by fairly high levels of gene flow historically and currently, thereby preventing substantial genetic divergence of regional cormorant populations.

Overall, rather than overt subspecific breaks between the traditional subspecies (Pacific, Interior/North Atlantic, and Southeast) we observed clinal variation of haplotypes and allele frequencies, wherein genetic dissimilarity increased with geographic distance. This pattern was evident in the range and distribution of  $\Phi_{ST}$  and  $F_{ST}$  values observed in the pairwise comparison matrices for all sites. In particular, the largest genetic distances were comparisons between the most geographically distant sites, while comparisons involving more centrally located sites were notably smaller and mostly not

significant. Specifically, the distal Pacific and North Atlantic sites had greater percent sequence divergence and differences in allele frequencies than the central Interior and Southeast sites. Secondly, the pattern was visualized by the PCA analysis. In particular, PC1 illustrated clinal differentiation and clearly discriminated sites according to a longitudinal gradient in allele frequencies, while PC2 and PC3 discriminated distal sites by the presence of unique or fixed alleles. Third, the STRUCTURE analysis identified three groups within North America, but individual genomes from across the U.S. and Canada were split between these groups suggesting substantial introgression between the purported subspecies. Nevertheless, the mean membership in cluster 1 relative to cluster 3 differed from east to west, for the breeding sites. Finally, a Mantel test showed a significant ( $P < 0.001$ ) and positive correlation between geographic and inter-individual genetic distance at microsatellite loci. Thus, genetic differences between sites and greater differences between the Pacific and North Atlantic sites are more likely due to geographic distance, rather than any discrete subspecific breaks.

The observed relationship between genetic and geographic distance is consistent with the isolation by distance model, which asserts that as geographic distance increases, genetic dissimilarity increases due to limited dispersal (Wright 1943). Further, peripheral populations are more likely to be genetically isolated than central populations as individuals at the center of a species' range can disperse in many directions, while those at the range edge can disperse in fewer directions (Eckert et al. 2008). This model is likely appropriate for the Double-crested Cormorant given their life-history traits, demographics, and observed movements. Cormorants are colonial nesters with mate

selection and pairing occurring at the breeding colony, a system conducive to high natal philopatry in stable colonies (Hatch 1995). Further, many young birds first breed where they were hatched and new colonies are thought to be formed by young birds at the closest suitable habitat to the natal colony (Hatch 1995). In fact, recoveries of banded birds have indicated high philopatry and site fidelity to areas near the natal colony (Dolbeer 1991, Clark et al. 2006). An analysis of band records east of the Rocky Mountains found that breeding age birds were recovered at a median and mean distance of 25 and 232 km, respectively, from their natal colony during the breeding season (Dolbeer 1991). Further, recoveries of birds banded in the Columbia River estuary as nestlings were all within 150 km of the estuary (Clark et al. 2006).

The genetic differentiation of the Pacific sites may be particularly influenced by limited dispersal and interchange with other regions. Clark et al. (2006) specifically noted that movements of cormorants banded in Oregon were nearly restricted to the Pacific Northwest, with only rare occurrences east of the Cascade-Sierra Nevada range, and likely did not extend to the Interior region. Dolbeer (1991) also noted a pronounced lack of interchange between cormorant colonies east of the Rocky Mountains and colonies in the Pacific. This suspected low level of interchange between regions and across a mountain range would also be facilitated by the lower density of colonies and individuals within the intermountain western states. In addition, Pacific cormorants tend to be residential while cormorants within other regions are migratory, spending the non-breeding season in the Gulf of Mexico and the southeast (Hatch 1995). Such differences in migratory tendencies may decrease gene flow between regions. It should be noted,

however, that the current distribution of cormorants in the western portion of the Interior region and the Southeast region is more extensive than the historical distribution (Wires and Cuthbert 2006). This range expansion may only increase gene flow between colonies and regions, further preventing genetic differentiation. In contrast, the North Atlantic lacks any perceivable barriers to gene flow and does not differ from the Interior and Southeast in migratory behavior. Thus, the observed differentiation of the North Atlantic population may be solely attributed to its location at the peripheral northeastern edge of the range, along with the assumed breeding site fidelity. To our knowledge, however, there have been no intensive studies of cormorant movements within the North Atlantic region.

Lack of a genetic discontinuity between the traditional geographic subspecies of Double-crested Cormorant does not necessarily imply strong demographic connectivity and homogenization across the range. Only a few dispersers per generation are necessary to prevent significant genetic differentiation (Wright 1931; Slatkin 1985, 1987; Mills and Allendorf 1996), but the same number of migrants may have no perceptible impact from a demographic standpoint. Further, populations may differ demographically despite gene flow. For example, although the Pacific population has exhibited continued gradual increases in total population size, the rate of increase is low relative to changes in the Interior population (Anderson et al. 2004, USFWS unpubl.data). In particular, colony declines have been documented over much of British Columbia, Washington, and Southern California despite substantial population growth in the Interior region (Anderson et al. 2004; Capitolo et al. 2004; Moul and Gebauer 2002; USFWS unpubl.



data). Likewise, growth of the North Atlantic population may have ceased by 1990 (Hatch and Weseloh 1999) with some areas exhibiting recent negative rates of change (Tyson et al. 1999). Thus, while the Pacific, Interior, North Atlantic, and Southeast populations are evidently connected by sufficient gene flow to prevent significant genetic divergence, the Pacific and North Atlantic may still be demographically distinct populations from those in the Interior and Southeast, particularly given tendencies towards regional differences in breeding site fidelity, demography, migratory habits, and the documented colony declines within the range of the Pacific and North Atlantic populations.

## **CONCLUSIONS**

Our mtDNA and microsatellite data provided the first comprehensive view of Double-crested Cormorant genetic structure. Consistent with current taxonomy, all analyses strongly supported a genetic distinction between Alaska and other populations, providing evidence for the continued recognition of an Alaskan subspecies (*P. a. cincinnatus*). In addition, analyses suggested the presence of another unique lineage within the Double-crested Cormorant. Specifically, southern California may represent the primary zone of introgression from a northward expansion of a southern lineage out of the species' range in northwestern Mexico. In contrast to current taxonomy, there was little genetic support for recognition of subspecies within the continental U.S. and Canada, outside of Alaska. Rather than genetically distinct populations corresponding to the traditional geographic

subspecies, we observed clinal variation of haplotypes and allele frequencies consistent with a pattern of gradual isolation by distance. This pattern implies that genetic differences across the range and greater differences between distal sites are due to geographic distance, rather than distinct subspecific breaks between regions. We note that while regions are evidently connected by sufficient gene flow to prevent significant genetic divergence, the Pacific and North Atlantic regions may still represent demographically distinct populations from the Interior and Southeast. Additional information on breeding site fidelity, natal philopatry, and movements throughout the annual cycle, would further clarify the demographic connectivity of these putative regional populations.

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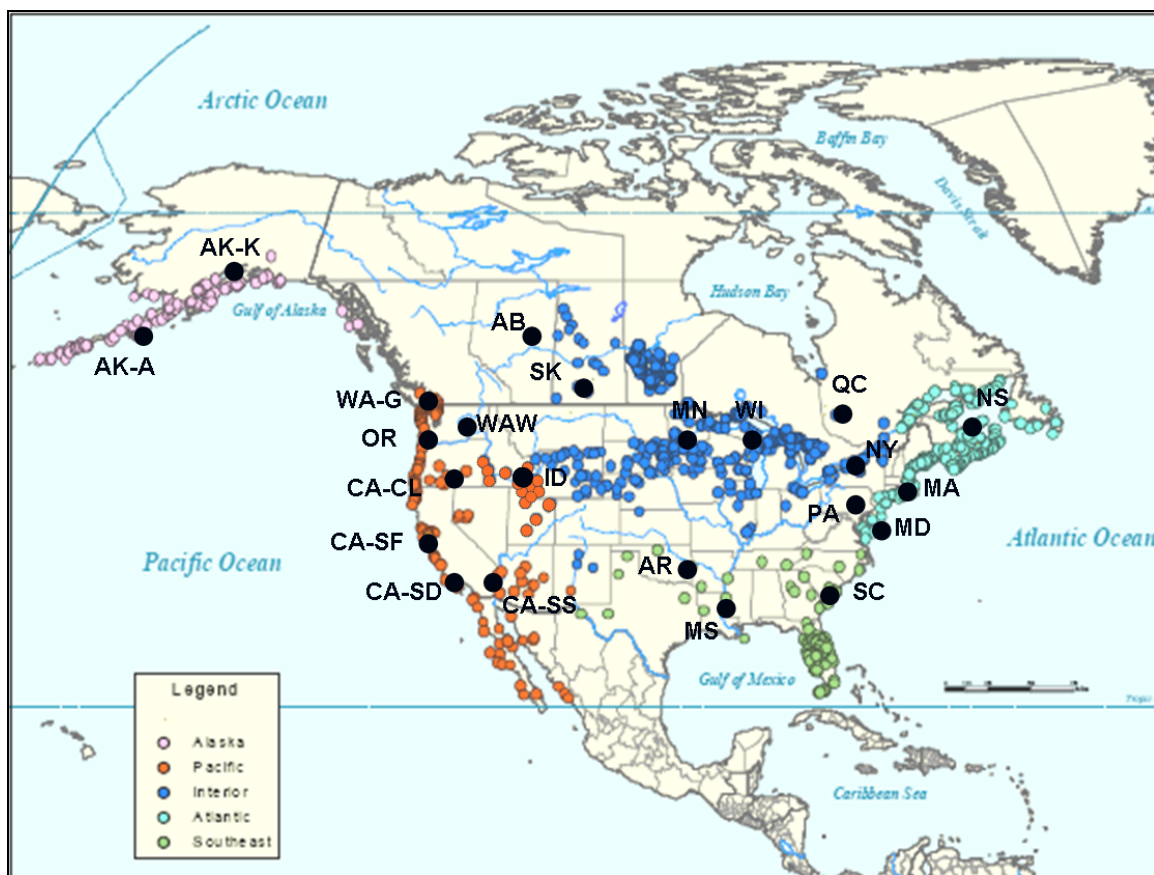
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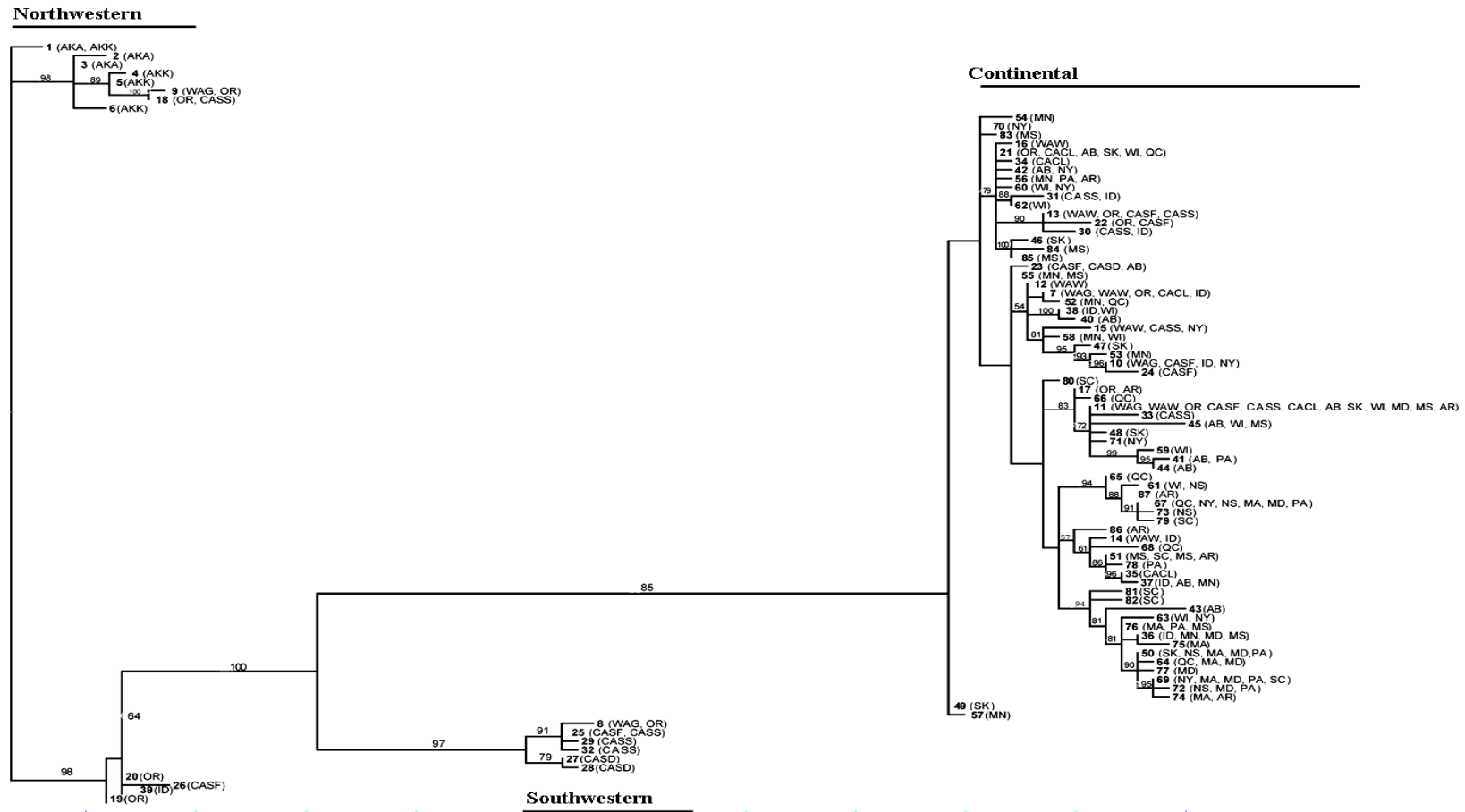
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Zink RM, Barrowclough GF, Atwood JL, Blackwell-Rago RC (2000) Genetics, taxonomy, and conservation of the threatened California gnatcatcher. *Conservation Biology*, **14**, 1394–1405.

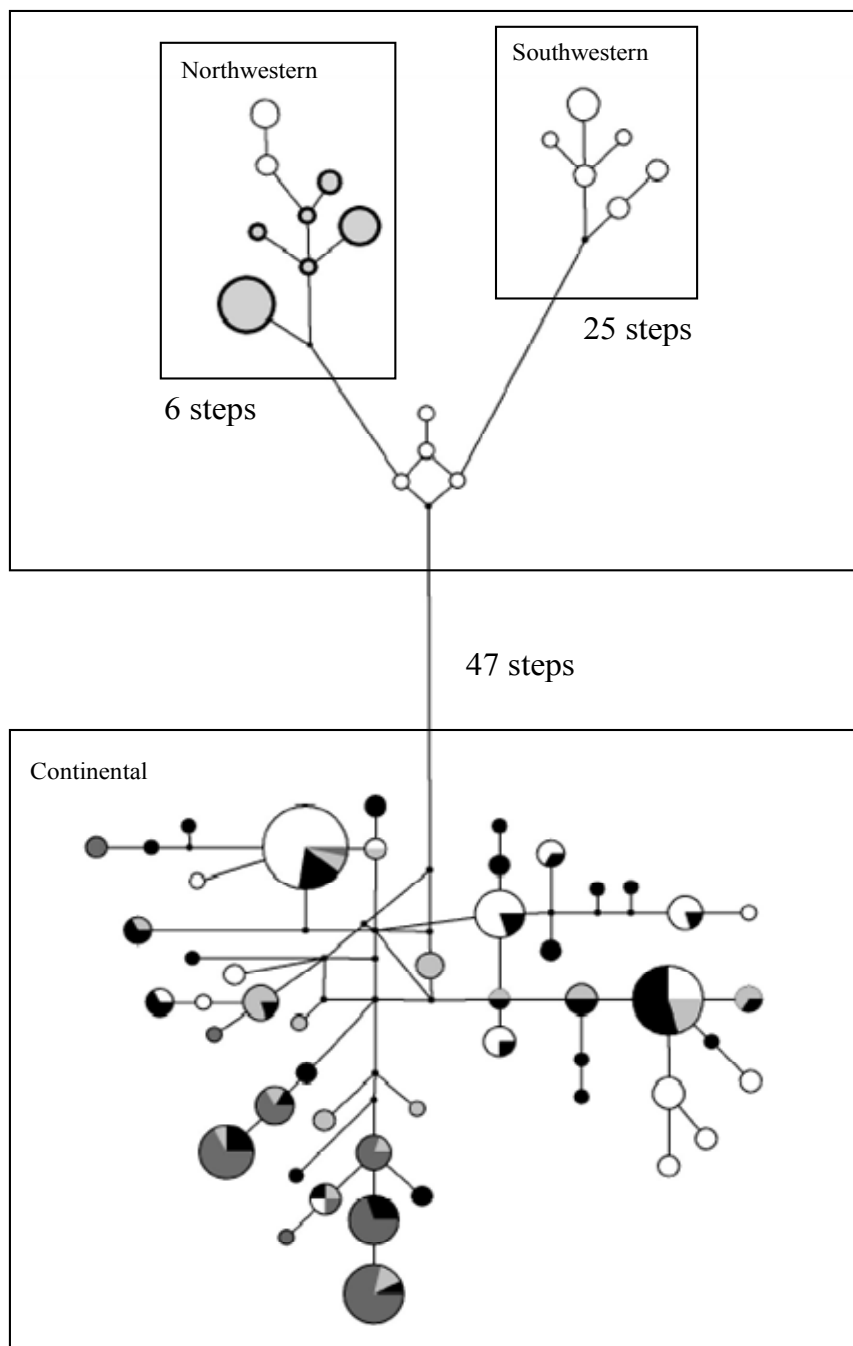
**Figure 3.1** Map of Double-crested Cormorant sampling sites included in the current study. Sample site information is provided in Table 3.1. Distributions of currently recognized subspecies are shown in pink (Alaska, *P. a. cincinnatus*), red (Pacific, *P. a. albociliatus*), green (Southeast, *P. a. floridanus*), and dark and light blue (Interior/North Atlantic, *P. a. auritus*). (Colony locations obtained from Wires and Cuthbert 2006; colony assignment to subspecies and region from Palmer 1962 and Hatch 1995).



**Figure 3.2** Maximum likelihood topology from analysis of mtDNA control region for Double-crested Cormorants. Haplotype numbers are shown in bold at branch tips and site codes (which correspond to codes used in Table 3.1 and Figure 3.1) are shown in parentheses. Numbers shown at nodes are Bayesian posterior probabilities from 100,000 samples trees. Outgroup taxa are not shown.

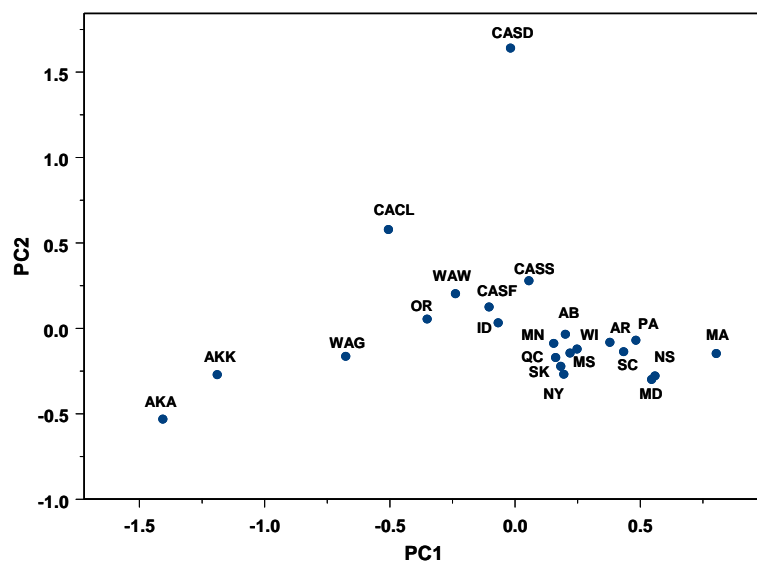


**Figure 3.3** Median joining network of mtDNA control region haplotypes for Double-crested Cormorants. Circle sizes are proportional to the number of individuals sharing the haplotype. Shades refer to the proportion of samples that came from a traditional subspecies: Alaska are in gray with a thick black outline; Pacific are in white; Southeast are in light gray; Interior/North Atlantic are in black (North Atlantic) and dark gray (Interior).

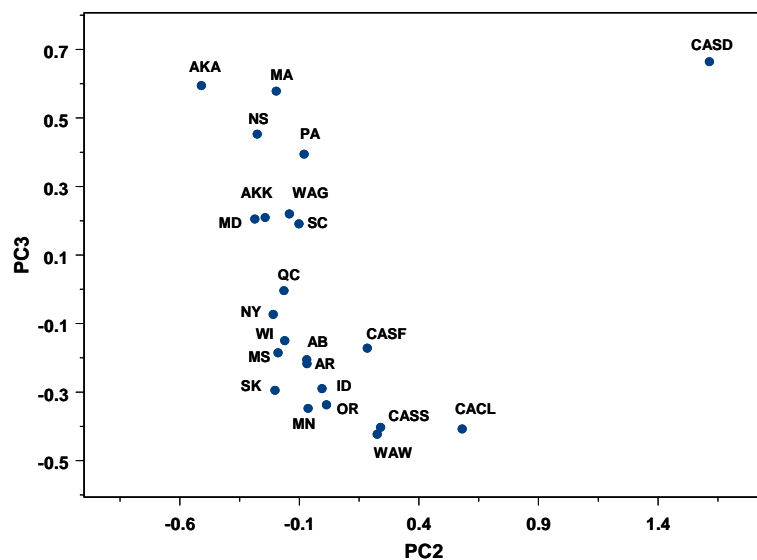


**Figure 3.4** (a) Principal component 1, which explained 21.3% of the variation in Double-crested Cormorant microsatellite allele frequencies versus PC2, which explained 12.7% of the variation; (b) PC3, which explained 9.2% of the variation versus PC2. Site codes correspond to codes used in Table 3.1.

(a)

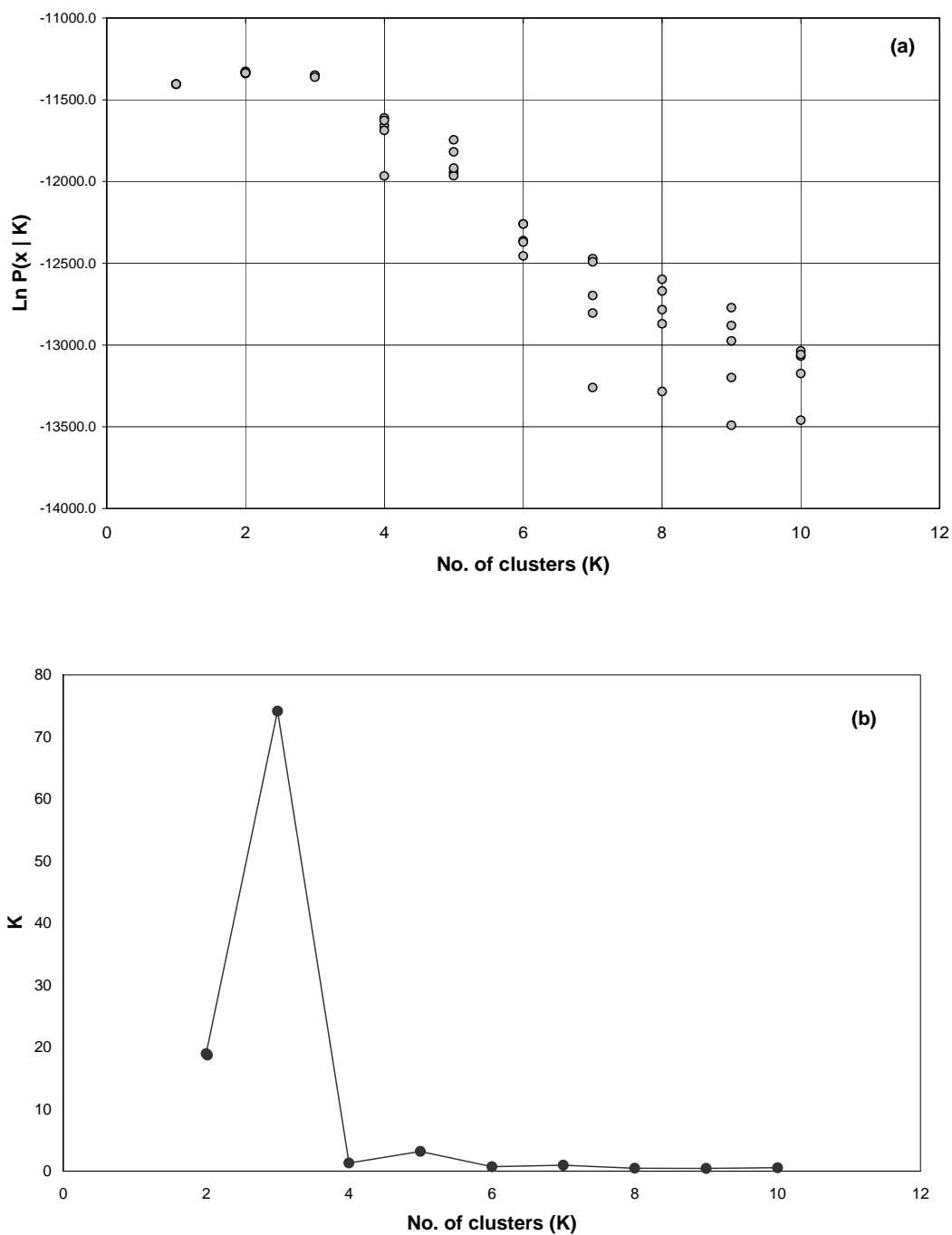


(b)

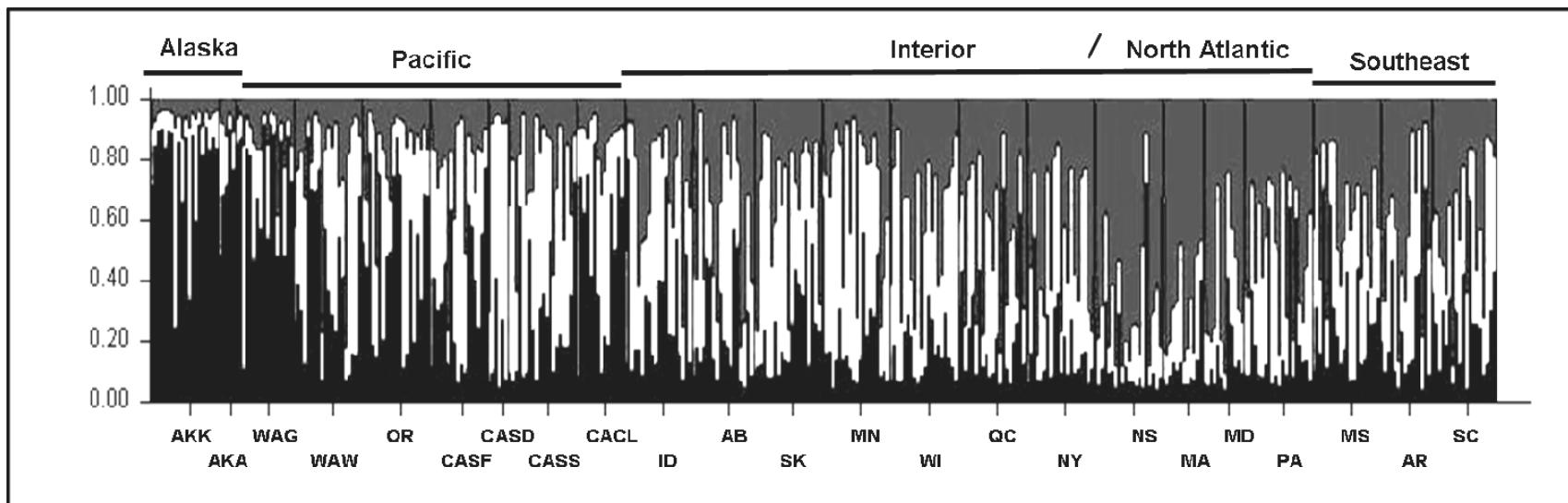




**Figure 3.5** Bayesian inference of the number of clusters ( $K$ ) of Double-crested Cormorants, based on 8 microsatellite loci.  $K$  was estimated using (a) the posterior probability of the data given each  $K$  (five replicates), and (b) the distribution of  $\Delta K$ .



**Figure 3.6** Population structure inferred by a Bayesian clustering algorithm implemented in STRUCTURE for 395 Double-crested Cormorants, based on 8 microsatellite loci. Three population clusters were identified: cluster 1 (gray); cluster 2 (white); and cluster 3 (black). The figure shows mean individual membership in each of these three clusters, with individuals grouped by subspecies and site. Labels above the figure refer to traditional subspecies designation; labels below the figure refer to the 23 sampled sites (see Table 3.1 for site codes).



**Table 3.1** Sample site information and within population genetic variation for mtDNA control region and eight microsatellite loci among 395 Double-crested Cormorants, grouped according to subspecies and breeding region.

Site code	Site location	mtDNA					Microsatellites			
		$N$	$h$	$s$	$\pi_n$	$Tajima's D$	$N$	$H_E$	$F_{IS}$	$R_S$
Alaska ( <i>P. a. cincinnatus</i> )										
AK-A	Eastern Aleutians, AK	5	0.700	8	0.0063	1.028	5	0.600	0.208	4.13
AK-K	Kenai Peninsula, AK	18	0.660	10	0.0067	2.122	20	0.593	0.094	3.87
Pacific ( <i>P. a. albociliatus</i> )										
WA-G	Gray's Harbor Co., WA	10	0.867	77	0.0553	1.725	17	0.611	0.061	4.21
WA-W	Walla Walla Co., WA	10	0.867	17	0.0078	-0.454	20	0.720	0.002	5.03
OR	Clatsop Co., OR	17	0.882	82	0.0418	0.631	20	0.668	0.065	4.79
CA-SF	San Francisco, CA	10	0.933	82	0.0383	-0.585	17	0.675	0.096	4.83
CA-SD	San Diego Co., CA	6	0.800	57	0.0459	1.398	6	0.608	0.007	4.15
CA-SS	Imperial Co., CA	10	1.000	80	0.0533	1.243	20	0.718	-0.008	5.23
CA-CL	Modoc Co., CA	10	0.756	13	0.0075	0.621	14	0.656	0.088	4.26
ID	Caribou Co., ID	10	0.978	69	0.0259	-1.393	20	0.680	-0.002	4.97
Interior/North Atlantic ( <i>P. a. auritus</i> )										
Interior										
AB	Lakeland, AB	10	1.000	31	0.0133	-0.752	18	0.712	0.054	5.16
SK	Regina, SK	8	0.964	22	0.0113	-0.409	20	0.709	0.069	4.85
MN	Cass Co., MN	10	1.000	26	0.0104	-1.022	20	0.683	0.075	5.02
WI	Door Co., WI	10	1.000	29	0.0125	-0.731	20	0.700	0.045	4.99
QC	Abitibi, QC	10	0.933	24	0.0115	-0.296	20	0.683	0.048	4.66
NY	Buffalo Co., NY	10	0.978	26	0.0127	-0.191	20	0.701	0.001	4.83
North Atlantic										
NS	Pictou Co., NS	10	0.822	13	0.0079	0.885	20	0.680	0.072	4.51
MA	Dukes Co., MA	10	0.911	14	0.0084	0.829	12	0.693	0.033	4.75
MD	Talbot Co., MD	10	0.933	17	0.0067	-1.073	12	0.732	0.047	5.11
PA	Harrisburg Co., PA	10	0.933	26	0.0108	-0.883	20	0.656	0.065	4.69
Southeast ( <i>P. a. floridanus</i> )										
AR	Sevier Co., AR	10	0.867	21	0.0105	-0.085	15	0.681	0.132	4.62
MS	Yazoo Co., MS	10	0.978	23	0.0113	-0.167	20	0.706	0.098	4.77
SC	Calhoun Co., SC	10	0.889	19	0.0008	-0.624	19	0.688	0.034	4.78

$N$  is the number of individuals analyzed,  $h$  is the haplotype diversity,  $s$  is the number of polymorphic sites,  $\pi_n$  is nucleotide diversity,  $H_E$  is gene diversity,  $F_{IS}$  is the within subpopulation inbreeding coefficient,  $R_S$  is the allelic richness.

**Table 3.2** Microsatellite primer sequences, PCR annealing temperatures (°C), product length (bp), number of alleles, and references for microsatellite primers included in this study of 395 Double-crested Cormorants.

Locus	Repeat Motif	Primer Sequence (5'-3')	Anneal T.	Length	Alleles	Reference
DCCO-01	(TCAC)19	F-GCTCAGTTGCTTTTCTCAC R-TTGCTGAATGGCTGTTCCA	54	191-251	13	New
DCCO-02	(TG)20	F-TAAAGCTAGGGTGATGGGC R-GCTGGAAATCTGGAAATCTCT	54	222-256	17	New
DCCO-03	(CA)14	F-TACAATAAGCACACAGAATTGC R-GCGTTTGTCCCATTCTGA	54	224-226	2	New
DCCO-04	(AG)18	F-CATTCACTACATCCATCTGC R-TTGGCAGTCTCAGTCACA	54	241-279	15	New
DCCO-05	(AC)9	F-ATCAGTCTGATGCATCCAGC R-TGCTGATGACAGAGTCTTCAG	54	224-230	2	New
PcD2	(GA)20	F-GATGGAAGTGAATAAAAGTTGG R-TTATGCAGAACTGAATTTTCC	52	186-218	17	Piertney et al. (1998)
PcT3	(GATA)28	F-CTTCCTGCTATGTCTATGCTTG R-ACAGCAAACAGCATCTATTCC	52	232-300	18	Piertney et al. (1998)
PcT4	(GATA)25	F-GGAGTCAGAGAACAACAACCAACC R-CAGCAGAGCGAGTTCTTTTAAC	52	301-397	28	Piertney et al. (1998)

**Table 3.3** Corrected average percent sequence divergence (i.e., net sequence divergence) between Double-crested Cormorants from different groupings at the mtDNA control region.

Comparison	Average % sequence divergence	<i>P</i>
<i>Between Subspecies</i>		
Alaska vs. Pacific	5.07	< 0.00001
Alaska vs. Interior/North Atlantic	8.00	< 0.00001
Alaska vs. Southeast	7.94	< 0.00001
Pacific vs. Interior/North Atlantic	0.53	< 0.00001
Pacific vs. Southeast	0.48	< 0.00001
Interior/North Atlantic vs. Southeast	0.02	0.12
<i>Between Regions</i>		
Alaska vs. All	6.72	< 0.00001
Pacific vs. Interior/North Atlantic/Southeast	0.52	< 0.00001
North Atlantic vs. Pacific/Interior/Southeast	0.44	< 0.00001
Interior vs. Pacific/North Atlantic/Southeast	0.12	< 0.00001
Southeast vs. Pacific/North Atlantic/Interior	0.10	< 0.00001
<i>Clades</i>		
Northwestern vs. Continental	7.98	< 0.00001
Southwestern vs. Continental	8.01	< 0.00001
Northwestern vs. Southwestern	3.96	< 0.00001

**Table 3.4** Pairwise  $\Phi_{ST}$  values between sites, listed by subspecies, at mtDNA control region for 395 Double-crested Cormorants. Significant values are shown in bold and highlighted.

Subspecies	Alaska		Pacific							Southeast			Interior/North Atlantic											
Site	AK-A	AK-K	WA-G	WA-W	OR	CA-SF	CA-SD	CA-SS	CA-CL	ID	AR	MS	SC	AB	SK	MN	WI	QC	NY	PA	MD	MA	NS	
AK-A	-																							
AK-K	-0.042	-																						
WA-G	0.378	<b>0.544</b>	-																					
WA-W	<b>0.917</b>	<b>0.920</b>	0.321	-																				
OR	<b>0.530</b>	<b>0.637</b>	0.014	0.113	-																			
CA-SF	<b>0.647</b>	<b>0.756</b>	0.055	0.049	-0.044	-																		
CA-SD	0.525	<b>0.685</b>	-0.001	0.602	0.230	0.262	-																	
CA-SS	0.463	<b>0.617</b>	-0.075	0.232	-0.018	-0.014	0.029	-																
CA-CL	<b>0.919</b>	<b>0.921</b>	0.328	-0.060	0.118	0.062	0.608	0.242	-															
ID	<b>0.759</b>	<b>0.828</b>	0.164	0.033	0.020	-0.057	0.403	0.089	0.049	-														
AR	<b>0.896</b>	<b>0.908</b>	<b>0.323</b>	0.061	0.130	0.072	0.584	0.235	0.022	0.024	-													
MS	<b>0.890</b>	<b>0.905</b>	0.313	0.063	0.128	0.069	0.570	0.229	0.036	0.012	-0.031	-												
SC	<b>0.911</b>	<b>0.916</b>	<b>0.330</b>	<b>0.228</b>	0.160	<b>0.137</b>	0.599	<b>0.268</b>	<b>0.222</b>	0.090	0.110	0.097	-											
AB	<b>0.876</b>	<b>0.899</b>	0.295	-0.038	0.111	0.041	0.551	0.210	-0.035	-0.005	0.001	-0.017	0.123	-										
SK	<b>0.893</b>	<b>0.908</b>	0.280	0.031	0.101	0.020	0.539	0.191	-0.006	-0.010	-0.034	-0.066	0.172	-0.024	-									
MN	<b>0.896</b>	<b>0.908</b>	0.302	0.059	0.126	0.049	0.564	0.217	0.068	-0.035	0.008	-0.018	0.163	-0.006	-0.034	-								
WI	<b>0.882</b>	<b>0.902</b>	0.304	-0.020	0.117	0.045	0.562	0.217	-0.024	-0.004	-0.030	-0.049	0.118	-0.065	-0.056	-0.023	-							
QC	<b>0.889</b>	<b>0.904</b>	0.315	0.114	0.138	0.097	0.580	0.247	0.108	0.043	0.030	0.027	0.001	0.053	0.075	0.079	0.026	-						
NY	<b>0.880</b>	<b>0.900</b>	0.301	0.030	0.122	0.043	0.559	0.217	0.033	-0.011	-0.030	-0.034	0.078	-0.015	-0.042	-0.018	-0.065	-0.020	-					
PA	<b>0.896</b>	<b>0.909</b>	<b>0.335</b>	0.251	0.179	0.149	0.585	<b>0.270</b>	<b>0.240</b>	0.099	0.116	0.080	0.040	0.123	0.153	0.162	0.125	0.024	0.073	-				
MD	<b>0.929</b>	<b>0.927</b>	<b>0.391</b>	<b>0.460</b>	0.250	<b>0.252</b>	<b>0.640</b>	<b>0.339</b>	<b>0.449</b>	<b>0.237</b>	<b>0.323</b>	0.280	0.209	0.317	0.357	<b>0.377</b>	<b>0.321</b>	0.178	<b>0.256</b>	-0.012	-			
MA	<b>0.915</b>	<b>0.919</b>	<b>0.378</b>	<b>0.422</b>	0.238	<b>0.235</b>	<b>0.626</b>	<b>0.328</b>	<b>0.419</b>	0.208	<b>0.280</b>	0.249	0.152	<b>0.295</b>	<b>0.330</b>	<b>0.337</b>	<b>0.281</b>	0.105	0.196	-0.006	-0.026	-		
NS	<b>0.917</b>	<b>0.920</b>	<b>0.381</b>	<b>0.435</b>	0.238	<b>0.241</b>	<b>0.633</b>	<b>0.331</b>	<b>0.439</b>	<b>0.230</b>	0.301	<b>0.313</b>	0.223	<b>0.328</b>	<b>0.373</b>	<b>0.371</b>	<b>0.281</b>	0.125	0.190	0.197	0.303	0.137	-	

**Table 3.5** Results from analysis of molecular variance (AMOVA) from mtDNA control region sequences and eight microsatellite loci with Double-crested Cormorant breeding sites grouped in different ways.

Groups	No. of groups	Variance Components	mtDNA		Microsatellites	
			% of variation	<i>P</i> -value	% of variation	<i>P</i> -value
All Sites	1	Among sites	47.62	0.000*	2.84	0.000*
		Within sites	52.38	0.000*	97.16	0.000*
Pacific	1	Among sites	12.53	0.003	2.25	0.000*
		Within sites	87.47	0.000*	97.75	0.000*
Interior/Atlantic and Southeast	1	Among sites	13.13	0.000*	1.47	0.000*
		Within sites	86.87	0.000*	98.53	0.000*
Subspecies	4	Among groups	48.09	0.000*	1.64	0.000*
		Among sites	6.95	0.001	1.71	0.000*
		Within sites	44.95	0.000*	96.66	0.000*
Alaska vs. California-San Diego vs. North Atlantic vs. rest of North America	4	Among groups	58.05	0.000*	2.71	0.000*
		Among sites	5.2	0.000*	1.68	0.000*
		Within sites	36.74	0.000*	95.61	0.000*
Alaska vs. California-San Diego vs. rest of North America	3	Among groups	71.68	0.000*	4.41	0.000*
		Among sites	4.74	0.000*	2.05	0.000*
		Within sites	23.58	0.000*	93.54	0.000*

\**P* < 0.00001

**Table 3.6** Pairwise  $F_{ST}$  values between sites, listed by subspecies, at eight microsatellite loci for 395 Double-crested Cormorants. Significant values are shown in bold and highlighted.

Subspecies	Alaska		Pacific							Southeast			Interior/North Atlantic										
Site	AK-A	AK-K	WA-G	WA-W	OR	CA-SF	CA-SD	CA-SS	CA-CL	ID	AR	MS	SC	AB	SK	MN	WI	QC	NY	PA	MD	MA	
AK-A	-																						
AK-K	0.040	-																					
WA-G	0.072	<b>0.049</b>	-																				
WA-W	<b>0.057</b>	<b>0.058</b>	<b>0.045</b>	-																			
OR	0.049	<b>0.048</b>	<b>0.045</b>	0.005	-																		
CA-SF	<b>0.060</b>	<b>0.065</b>	<b>0.052</b>	-0.002	0.010	-																	
CA-SD	0.149	<b>0.127</b>	<b>0.105</b>	0.032	<b>0.052</b>	0.035	-																
CA-SS	<b>0.061</b>	<b>0.070</b>	<b>0.056</b>	-0.001	0.001	0.002	0.032	-															
CA-CL	<b>0.081</b>	<b>0.045</b>	<b>0.051</b>	0.010	0.013	0.023	0.037	0.020	-														
ID	<b>0.061</b>	<b>0.061</b>	<b>0.057</b>	0.006	0.006	0.013	<b>0.061</b>	0.005	0.022	-													
AR	<b>0.097</b>	<b>0.106</b>	<b>0.066</b>	0.021	<b>0.030</b>	<b>0.025</b>	<b>0.067</b>	0.014	<b>0.045</b>	0.025	-												
MS	<b>0.067</b>	<b>0.071</b>	<b>0.052</b>	0.006	0.018	0.019	<b>0.057</b>	0.010	0.026	0.021	0.013	-											
SC	0.060	<b>0.073</b>	<b>0.060</b>	0.019	<b>0.031</b>	<b>0.025</b>	<b>0.059</b>	<b>0.019</b>	0.037	0.027	0.024	0.008	-										
AB	0.049	<b>0.078</b>	<b>0.068</b>	0.002	0.016	0.009	0.040	0.001	0.030	0.008	0.005	0.009	0.012	-									
SK	<b>0.061</b>	<b>0.076</b>	<b>0.067</b>	0.003	0.020	0.016	<b>0.067</b>	0.008	0.027	0.005	0.009	0.005	0.019	-0.004	-								
MN	0.058	<b>0.077</b>	<b>0.071</b>	0.012	0.008	0.019	0.045	-0.005	0.030	0.004	0.013	0.012	0.014	-0.003	0.008	-							
WI	<b>0.065</b>	<b>0.065</b>	<b>0.067</b>	0.009	0.021	0.013	<b>0.056</b>	0.009	<b>0.032</b>	0.023	0.023	0.005	0.014	0.004	0.005	0.010	-						
QC	0.062	<b>0.058</b>	<b>0.056</b>	0.013	0.018	0.025	<b>0.062</b>	0.015	<b>0.031</b>	0.012	0.011	-0.001	0.009	0.005	0.007	0.006	0.006	-					
NY	<b>0.069</b>	<b>0.075</b>	<b>0.057</b>	0.010	<b>0.021</b>	<b>0.022</b>	0.052	0.014	0.027	0.027	0.018	-0.001	0.012	0.005	0.011	0.009	-0.004	0.000	-				
PA	<b>0.105</b>	<b>0.093</b>	<b>0.055</b>	0.020	<b>0.044</b>	0.024	<b>0.062</b>	<b>0.036</b>	<b>0.052</b>	<b>0.034</b>	0.031	0.016	0.027	<b>0.027</b>	<b>0.028</b>	<b>0.043</b>	0.020	<b>0.013</b>	0.026	-			
MD	0.089	<b>0.094</b>	<b>0.071</b>	0.019	<b>0.044</b>	0.034	0.073	0.026	<b>0.058</b>	<b>0.046</b>	0.029	0.006	0.005	0.014	0.023	<b>0.036</b>	0.005	0.006	0.000	0.013	-		
MA	<b>0.106</b>	<b>0.107</b>	0.057	0.015	<b>0.035</b>	0.027	0.057	0.014	<b>0.059</b>	<b>0.033</b>	0.026	0.007	0.005	0.013	0.024	0.019	0.010	0.010	0.004	0.002	-0.013	-	
NS	<b>0.071</b>	<b>0.095</b>	<b>0.073</b>	<b>0.039</b>	<b>0.044</b>	<b>0.039</b>	<b>0.086</b>	<b>0.037</b>	<b>0.060</b>	<b>0.045</b>	<b>0.036</b>	<b>0.024</b>	0.006	0.023	<b>0.032</b>	<b>0.035</b>	0.021	0.017	0.015	<b>0.034</b>	-0.003	-0.005	



## Chapter 4. Conclusions

### 4.1 *Summary*

This thesis provides the first wide-ranging assessment of the phylogeography and population genetic structure of the Double-crested Cormorant. It further resulted in the first microsatellite markers developed specifically for a North American member of the avian order Pelecaniformes. The mitochondrial and microsatellite data provide a comprehensive assessment of the four putative subspecies described for the species. Given the highly varying conservation and management status of Double-crested Cormorants throughout their range in North America, results of the study provide guidance for management practices on their behalf.

### 4.2 *Marker Development*

The *Phalacrocorax* is a genetically understudied genus in a genetically understudied order, Pelecaniformes. Consequently, there is a pronounced lack of molecular markers developed for cormorants. This study described the isolation and characterization of six novel microsatellite loci for the Double-crested Cormorant. These loci should prove useful for further genetic studies of the Double-crested Cormorant, as well as other members of the genus *Phalacrocorax*, given their high levels of polymorphism and demonstrated cross-amplification.

#### 4.3 Taxonomic Evaluation and Regional Differentiation

Genetic data provided limited support for current taxonomy.

- Consistent with current taxonomy, the mtDNA and microsatellite data provided strong evidence that the Alaska subspecies is genetically divergent from other Double-crested Cormorant populations in North America.
- In addition, our data suggested strong genetic divergence in the southwestern U.S.; southern California may represent a zone of introgression resulting from a northward expansion of a unique lineage from the species' range in northwestern Mexico.
- In contrast, there was little support for recognition of subspecies within the continental U.S. and Canada, outside of Alaska. Rather than genetically distinct regions corresponding to the described subspecies [*P. a. albociliatus* (Pacific), *P. a. auritus* (Interior and North Atlantic), and *P. a. floridanus* (Southeast)] we observed a distribution of genetic variation consistent with a pattern of gradual isolation by distance. This pattern implies that genetic differences across the range are due to geographic distance, rather than discrete subspecific breaks between regions.

#### 4.4 Phylogeography and Population Genetic Structure

Phylogenetic analyses identified three primary genetic subsets: (1) a large clade including haplotypes from across the range, (2) a unique lineage primarily restricted to Alaska, and (3) a unique lineage associated with the southwestern limit of the species' range. The

combined analyses of the eight microsatellite loci corroborated the major findings of the mitochondrial data. Population genetic analyses of both markers were congruent and supported the inferred phylogenetic structure. Specifically, both data sets identified Alaska as being the most genetically distinct region within the range of the Double-crested Cormorant, while illustrating a pattern of gradual isolation by distance across the rest of the range in North America.

#### *4.4 Conservation Implications*

- Historical records, contemporary population estimates, and field observations are consistent with recognition of the Alaskan subspecies as distinct and potentially of conservation interest. Specifically, Alaska currently has the smallest population size of the regions examined, this contemporary population is less numerous and widespread than historically, and cormorants in the region may be experiencing declines.
- A genetic discontinuity in the southwestern portion of the species' range has not been described previously, and additional study will be necessary to describe the genetic and geographic status of this potentially unique lineage.
- Although the Pacific, North Atlantic, Interior, and Southeast regions are evidently connected by sufficient gene flow to prevent significant genetic divergence, the Pacific and North Atlantic regions may still represent demographically separate populations from the Interior and Southeast, particularly given tendencies towards breeding site fidelity, demographic

differences between regions, and the documented population declines at some colonies within the Pacific and North Atlantic regions.

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