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ABSTRACT

Species complexes that have undergone recent radiations are often characterized by extensive allele sharing due to recent ancestry and (or) introgressive hybridization. This can result in discordant evolutionary histories of genes and heterogeneous genomes, making delineating species limits difficult. Here we examine the phylogenetic relationships among a complex group of birds, the white-headed gulls (Aves: Laridae), which offer a unique window into the speciation process due to their recent evolutionary history and propensity to hybridize. Relationships were examined among 17 species (61 populations) using a multilocus approach, including mitochondrial and nuclear intron DNA sequences and microsatellite genotype information. Analyses of microsatellite and intron data resulted in some species-based groupings, although most species were not represented by a single cluster. Considerable allele and haplotype sharing among white-headed gull species was observed; no locus contained a species-specific clade. Despite this, our multilocus approach provided better resolution among some species than previous studies. Interestingly, most clades appear to correspond to geographic locality: our BEAST analysis recovered strong support for a northern European/Icelandic clade, a southern European/Russian clade, and a western North American/*canus* clade, with weak evidence for a high latitude clade spanning North America and northwestern Europe. This geographical structuring is concordant with behavioral observations of pervasive hybridization in areas of secondary contact. The extent of allele and haplotype sharing indicates that ecological and sexual selection are likely not strong enough to complete reproductive isolation within several species in the white-headed gull complex. This suggests that just a few genes are driving the speciation process.

1. Introduction

Speciation is a continuous process (Mallet and Dasmahapatra, 2012): as populations diverge, they are expected to evolve through intermediate states of reproductive isolation (i.e., incipient speciation). The rate at which species advance through these intermediate states is influenced by both genetic (selection, lineage sorting, genetic drift) and behavioral (mate choice) processes (Nosil et al., 2009). Among recently evolved species, trait differences alone may not be strong enough to complete the speciation process, as trait discrimination among conspecifics is a driving factor in premating isolation (Nosil et al., 2009; Bank et al., 2012; Hudson and Price, 2014). Propensity for dispersal and subsequent hybridization may reduce the rate of lineage sorting among recently isolated species, thereby prolonging and in some cases reversing the speciation process (Webb et al., 2011). Reinforcement via assortative mating can expedite speciation in areas of secondary contact by reducing gene flow and promoting genetic divergence among species (Kondrashov and Shpak, 1998; Ortiz-Barrientos et al., 2004; Hoskin et al., 2005). Speciation can be

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further expedited in spite of prevailing gene flow, if traits arising from adaptive divergence also promote assortative mating (e.g., magic traits; Servedio et al., 2011), thereby resulting in divergence among taxa restricted to few genomic markers (i.e., genomic islands; Nosil et al., 2009; Ellegren et al., 2012). Indeed, limited genomic differences except at loci controlling selective characteristics have been reported between phenotypically distinct taxa that mate randomly at hybrid zones (Counterman et al., 2010; Nadeau et al., 2012; Poelstra et al., 2014).

Climatic oscillations occurring throughout the Pleistocene shaped the evolution and distribution of species (Hewitt, 2004a). During glacial maxima, species ranges contracted to Arctic and southern refugia with lineages diverging in allopatry. Expansion to ice-free areas during interglacial periods resulted in divergent lineages coming into secondary contact and, in some instances, hybridizing. Patterns of expansion and contraction and zones of secondary contact varied by region: the extent of glacial ice sheets was greater in North America than Europe, the Bering Land bridge connected Alaska with Siberia, and geographical features differ between North America and Europe (Hewitt, 2004a). Species restricted to high latitude refugia experienced fewer glacial oscillations; Holarctic species show shallow but clear phylogenetic structure (Hewitt, 2004a). Conversely, species occupying more temperate regions were able to retain higher levels of genetic diversity and form distinct geographic lineages with deeper divergence than their northern counterparts (Hewitt, 2004b).

“White-headed” gulls (*sensu* Pons et al., 2005; *Larus* spp.: Aves: Charadriiformes: Laridae) present an interesting case study of speciation due to their recent evolution and tendency to hybridize (Liebers et al., 2001; Crochet et al., 2003; Gay et al., 2007, 2008; Sternkopf et al., 2010; Sonsthagen et al., 2012; Pons et al., 2014). The white-headed gull clade is represented by 21–23 species (depending on taxonomic authority), most of which (17–19 species) are endemic to the Northern Hemisphere (Fig. 1; Olsen and Larsson, 2004; BirdLife International and NatureServe, 2015). Several species have large breeding distributions that span the Holarctic (e.g., *argentatus*, *canus*, and *hyperboreus*), Palearctic (*cachinnans*, *fuscus*, and *michahellis*), Nearctic (*delawarensis*), and coastal areas of the North Atlantic (*marinus*) and North Pacific (*glaucescens* and *schistisagus*). Species with more restricted distributions are present largely within western North America (*californicus*, *heermanni*, *livens*, and *occidentalis*) and the high Canadian Arctic and Greenland (*glaucoides* and *thayeri*).

Higher level phylogenetic relationships within the Charadriiformes (Ericson et al., 2003; Fain and Houde, 2007) and Laridae (Pons et al., 2005) are fairly well resolved, but determining relationships among white-headed gulls has proven difficult. For example, phylogenetic reconstructions based on two mitochondrial genes (Crochet et al., 2000; Pons et al., 2005) provided limited phylogenetic resolution, despite morphological differences among largely sympatric species. Lack of phylogenetic resolution within the white-headed gull complex has been attributed to the recent evolutionary history of this clade (Pons et al., 2005). The propensity of members of this complex to hybridize further complicates reconstructions of phylogenetic relationships (Sternkopf et al., 2010). Recent range expansions have resulted in hybridization between resident and colonist species (resident *hyperboreus* with colonist *argentatus*, Iceland, ca. 1925, Vigfúsdóttir et al., 2008; resident *argentatus* with colonist *cachinnans*, Poland, ca. 1980s, Neubauer et al., 2009). In areas of secondary contact, viable hybrid offspring and stable hybrid zones are often produced (*glaucescens*–*occidentalis*; Good et al., 2000; Gay et al., 2008). Hybridization events have resulted in introgression: *argentatus* and *hyperboreus* breeding at high latitudes were more genetically similar to populations in close geographic proximity regardless of species designation than to populations elsewhere within the same species (Sonsthagen et al., 2012). Moreover, allele sharing at both mitochondrial and nuclear loci in Europe cannot be explained by incomplete sorting of ancestral polymorphisms alone (Crochet et al., 2003). Comparisons of introgression rates between molecular and morphological markers, however, suggest that assortative mating based on phenotypic traits, as well as differences in breeding phenology, may act as premating barriers (*argentatus*–*cachinnans*; Gay et al., 2007; Neubauer et al., 2009). Regardless, hybridization events involving most members of the white-headed gull complex have been reported (Olsen and Larsson, 2004) and in some areas hybridization is so pervasive that intermediate phenotypes dominate the colonies (*argentatus*–*glaucescens*, Cook Inlet, Alaska, Williamson and Peyton, 1963; *glaucescens*–*hyperboreus*, Nunivak Island, Alaska, Swarth, 1934), indicating that premating isolating mechanisms are not strong enough to maintain species boundaries in sympatry.

Phylogenetic relationships of recently derived species, especially species with limited levels of reinforcement and the propensity to hybridize, can be difficult to resolve, as incomplete lineage sorting and introgressive hybridization can result in heterogeneous genomes and discordant evolutionary histories among loci (Carstens and Knowles, 2007). Here we employed a multi-individual and multi-locus approach to examine species relationships among 16 of the 23 species of white-headed gulls. We assayed markers (nuclear introns, mitochondrial control region, and microsatellite loci) that differ in their overall mutation rate and effective population size, in an attempt to disentangle phylogenetic relationships among taxa. Microsatellite repeat units and nucleotide substitutions (mtDNA and nuclear introns) differ in their primary mutation mechanism, microsatellite loci providing greater resolution at more recent evolutionary scales due to their faster mutation rate (Degnan et al., 1999; Wang, 2010). Mitochondrial DNA has a smaller effective population size and shorter coalescent time than nuclear loci (Johnson and Clayton, 2000), whereas nuclear introns do not saturate significantly in avian species until they have diverged for 50 Myr (Moore et al., 1999), likely retaining more historical phylogenetic information than mitochondrial loci. Therefore, if the recent evolutionary history of this complex (i.e., incomplete lineage sorting) is the main factor in the lack of phylogenetic resolution in white-headed gulls, we would expect nuclear introns and potentially mtDNA to exhibit weak, if any, differentiation, whereas the microsatellite loci would uncover species clusters. Conversely, if introgressive hybridization is pervasive, then we would expect admixture across all marker types and that species would cluster by locality. Sampling several types of markers and many individuals per species is especially important for species for which hybridization is common, such as the white-headed gulls, as single representative of species and single locus approaches may have increased chance of sampling introgressed alleles or haplotypes, thereby misleading phylogenetic inference (see Peters et al., 2005; Jacobsen and Omland, 2011).

2. Materials and methods

2.1. Taxon sampling

Tissue samples of breeding-season adults ($n = 658$), representing 17 species and 61 populations of white-headed gulls, were collected or obtained through loans. Species and subspecies represented were *L. argentatus* (*L. a. argentatus*, *L. a. argenteus*, *L. a. smithonianus*, and *L. a. vegae*), *L. cachinnans* (including *L. c. mongolicus*), *L. californicus*, *L. canus*, *L. delawarensis*, *L. dominicanus*, *L. fuscus*, *L. glaucescens*, *L. glaucoides*, *L. heermanni*, *L. hyperboreus*, *L. livens*, *L. marinus*, *L. michahellis*, *L. occidentalis* (*L. o. occidentalis* and *L. o. wymani*), *L. schistisagus*, and *L. thayeri* (Supplementary

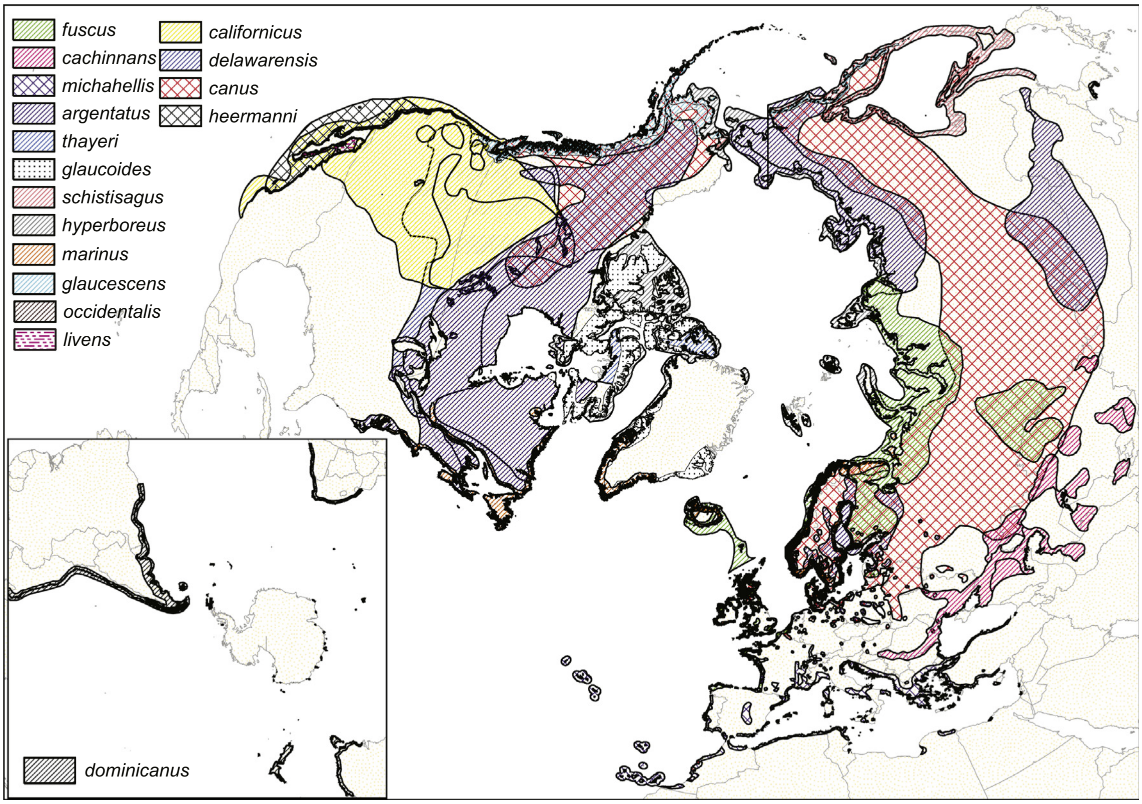

Fig. 1. Breeding and year-round distributions (BirdLife International and NatureServe, 2015) of 17 species of 'white-headed' gulls (*Larus* sp.). Each species has a unique color or pattern.

Table S1). We used a black-headed gull species, *Leucophaeus pipixcan* ($n = 3$), as outgroup for the phylogenetic analyses (Supplementary Table S1). Because of the limited number of breeding individuals of *thayeri* in tissue collections, non-breeding adults were also included in this study. Care was taken to ensure that plumage characteristics of the sampled individuals were consistent with pure species, given the tendency for hybridization in this group (Pierotti, 1987; Olsen and Larsson, 2004 and citations therein). Species classifications follow the American Ornithologists' Union *Check-list of North American Birds* (1998) and the relevant supplement (Banks et al., 2008); individuals were diagnosed to subspecies based on morphological characteristics and geographical origin (Olsen and Larsson, 2004).

2.2. Laboratory techniques

DNA was extracted using an AutoGen animal tissue extraction kit (AutoGen, Holliston, Maine). Genomic DNA concentrations were quantified using spectrophotometry and diluted to 50 ng μL^{-1} working solutions. Multilocus genotypes were collected at 11 microsatellite loci containing dinucleotide repeat motifs: Hg16, Hg18, Hg25 (Crochet et al., 2003), K16 (Tirard et al., 2002), LarZAP12, LarZAP19, LarSNX24, LarZAP26 (Gregory and Quinn, 2006), Rbg13, Rbg18, and Rbg29 (Given et al., 2002). PCR amplifications followed Sonsthagen et al. (2007) with two modifications. The forward primer was end-labeled with one of two fluorescent phosphoramidite dyes (FAM or HEX). Fluorescently labeled PCR products were electrophoresed on an automated DNA sequencer (ABI 3130XL; Applied Biosystems, Foster City, California) and sized using GENEMAPPER® version 4.0 (Applied Biosystems, Foster City, California) with a universal ROX-labeled size standard (DeWoody et al., 2004). Ten percent of the samples were amplified and sized in duplicate for quality control purposes. No inconsistencies in genotypes were observed between replicates. Microsatellite genotype data are accessioned at the USGS Alaska Science Center data repository (http://dx.doi.org/10.5066/F74X55WS).

Six nuclear introns were sequenced: α-enolase intron 8 (using primers EnolL731 and EnolH912; Friesen et al., 1997), Ghrelin (Ghrel) intron 3 (Ghrel3F and Ghrel4R; Sonsthagen et al., 2012), ornithine carboxylase (OD) intron 7 (OD7F and OD8R; Sonsthagen et al., 2007), clathrin heavy-chain (CHC) intron 5 (CHC5F and CHC6R; Sonsthagen et al., 2012), myelin proteolipid protein (MPP) intron 4 (MPP4.F and MPP5.R; Friesen et al., 1999), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) intron 11 (G3P13F from Van Tuinen et al., 2001, and GAPDH.12Rev from Sonsthagen et al., 2012). Ghrel had two recurring insertion/deletions (indels). To obtain sequence information from the entire fragment for individuals that were heterozygous for both indels, two internal sequencing primers were developed (Ghrel-IntF; Ghrel-IntR; Sonsthagen et al., 2012). We amplified a 2500 bp fragment of the mitochondrial genome, which included the control region, ND6, and part of 16S rRNA, using primer pairs L15522 (Sonsthagen et al., 2012) and H1816 (Helbig and Seibold, 1999). From this, we directly sequenced 430 bp of domain I of the control region using sequencing primers H419 and H519 (following Liebers et al., 2001), because of the presence of nuclear pseudogenes in this group. PCR amplifications followed Sonsthagen et al. (2012). Nuclear intron sequences that contained double peaks of approximately equal peak height, indicating the presence of two alleles, were coded with International Union of Pure and Applied Chemistry (IUPAC) degeneracy codes and treated as polymorphisms. Sequences were reconciled using Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, Michigan). Ten percent of the samples and individuals with the appearance of introgressed haplotypes were sequenced in duplicate for quality control purposes. No inconsistencies in sequences were observed between replicates.

Sequences are accessioned in GenBank (accession numbers JQ708216–JQ710335; KX520709–KX522538).

2.3. Genetic diversity and structure analyses

Allelic phases of nuclear introns were inferred from diploid sequence data using PHASE 2.0 (Stephens et al., 2001) with all samples analyzed in a single dataset. PHASE uses a Bayesian approach to reconstruct haplotypes from population genotypic data and allows for recombination and the decay of linkage disequilibrium with distance. The PHASE analysis (1000 iterations with a 1000 burn-in period) was repeated three times; results were consistent across runs. Each locus was analyzed separately. We calculated allelic frequencies and expected and observed heterozygosities for each microsatellite locus and the six nuclear introns using GenePop version 4.2 (Raymond and Rousset, 1995). Hardy-Weinberg equilibrium (HWE) was tested in GenePop for microsatellite and nuclear intron loci, adjusting for multiple comparisons using Bonferroni corrections ($\alpha = 0.05$). Haplotype and nucleotide diversity were estimated for the mtDNA data in Arlequin 3.11 (Schneider et al., 2000).

A discriminant analysis of principal components (DAPC) was performed on the genotypic nuclear data (microsatellite and intron), retaining population information, using the *adegent* package in R3.2.2 (Jombart et al., 2010). The optimal number of retained principal components was determined using the function *optim.a.score*. We retained 25 principal components that explained 72.6 and 79.5% of the variation for the microsatellite and nuclear intron data sets, respectively. The first two principal components were plotted to illustrate the relationships among individuals.

Genotypic nuclear data (microsatellite and intron) also were analyzed in STRUCTURE 2.2.3 (Pritchard et al., 2000) to detect the occurrence of population structure without *a priori* knowledge of putative populations. A series of analyses were performed (1) among all gull individuals, and (2) within each cluster identified in analysis 1 to detect fine-scale partitions in genetic variation (see Evanno et al., 2005). Microsatellite and nuclear intron data were analyzed separately, because the mutation mechanism differs between marker types. Data were analyzed using an admixture model assuming correlated frequencies to probabilistically assign individuals to putative populations (parameters: burn-in 50,000 iterations; 500,000 Markov chain Monte Carlo iterations) with the number of possible clusters (*K*) ranging from 1 to 10. Analyses were repeated ten times and were consistent across runs. We used the method of Evanno et al. (2005) and the *K* that maximized the likelihood to determine the most likely number of clusters given the data.

2.4. Phylogenetic analyses

Phylogenetic relationships were reconstructed using sequences from the six nuclear introns and the mtDNA control region. Analyses were conducted on a reduced data set: each taxon (species, subspecies, or population) was represented by ten randomly chosen individuals having the complete multi-locus data set, except for *cachinnans* from Ukraine ($n = 7$), for which we were unable to obtain sequence data at all loci for 10 samples. Trees were constructed using three approaches: maximum likelihood (ML) using RAxML (Stamatakis et al., 2008), maximum parsimony (MP) as implemented in PAUP* v4b10 (Swofford, 2003) and Network 4.6.1.2 (Fluxus Technology Ltd., 2014), and Bayesian methods using *BEAST 1.7.5 (Heled and Drummond, 2010; Drummond et al., 2012) and MrBayes 3.2 (Ronquist et al., 2012). Model selection for Bayesian analyses was based on likelihood ratio tests using the Akaike Information Criterion (Supplementary Table S2; Modeltest 3.7; Posada and Buckley, 2004).

Nodal support for the unpartitioned ML analysis was assessed based on 100 bootstrap replicates. Two unweighted MP analyses were conducted, treating indels as (1) missing data or (2) a new state. A heuristic search was employed with 100 random addition sequences. Bootstrapping was conducted using 500 replicates, each with 10 random addition sequences. A consensus tree was generated from 1 million trees incorporating tree weights. In addition, haplotypic and allelic networks for mtDNA and individual nuclear introns were constructed in NETWORK using the Median Joining network (Bandelt et al., 1995) on the full data set.

The Bayesian analysis in MrBayes used Markov-chain Monte Carlo (MCMC) sampling, and was run for 5 million generations with six simultaneous runs and seven chains, sampling trees every 1000 generations on (1) mtDNA only and (2) mtDNA and nuclear introns. The program Tracer v1.4 (Rambaut and Drummond, 2007) was used to examine mixing and convergence; effective sample sizes (ESS) were large for parameters estimated in the MCMC, and a burn-in of one million generations was used. We allowed model parameters to be unlinked across partitions, with each partition corresponding to a different gene, and parameters varied among the partitions: substitution rates, shape parameter of the gamma distribution of substitution rates, stationary base frequencies, proportion of invariable sites, and nucleotide substitution ratio.

Lastly, the species tree was estimated in *BEAST 1.7.5, which provides joint inferences of the species tree and individual gene trees, using the reduced data set. The best-fit model for each gene partition was established using Modeltest. Gene site substitution models, clock models, and topology were estimated unlinked. The Yule process was used as the species tree prior, and population size was piecewise linear and constant root. The clock model was set as a random local clock (Drummond and Suchard, 2010) for all of the genes. We did not estimate divergence times. A rate of substitution of 1 was fixed for the mtDNA control region and the substitution rates for the remaining genes were estimated relative to it. Two independent runs of 500 million generations were performed, sampling once every 2000 generations. Again, Tracer was used to examine mixing and convergence. Posterior probabilities (pp) of the nodes were computed after a 10% burn-in.

3. Results

3.1. Genetic diversity and structure

Multilocus microsatellite genotypes were collected from 658 individuals representing the 17 species. Observed heterozygosity among taxa and populations ranged from 22.0 to 71.1% (Supplementary Table S3). Most populations were in HWE after applying the Bonferroni correction; populations that deviated from HWE were heterozygote deficient (Supplementary Table S3). Nuclear introns were 323–665 bp in length and contained 21–57 variable sites. PHASE reconstructed 31–160 alleles for the individual introns (Fig. 2A–F). Probabilities of reconstructed haplotypes ranged primarily from 0.80 to 1.00, although a minority of individuals had probabilities ranging from 0.50% to 0.78% (5.5% of individuals for CHC, 3.5% for enolase, 12.3% for GAPDH, 1.3% for Ghrel, 13.1% for MPP, and 2.1% for OD). Observed heterozygosities ranged from 17.6% to 77.3% (Supplementary Table S3). The mtDNA control region was 430 bp long and contained 81 variable sites among 235 unique haplotypes (Fig. 2G). Number of haplotypes per population ranged from 2 to 14 (Supplementary Table S3). Haplotype (h) and nucleotide (π) diversity were high for most populations, with values ranging from 0.286 to 1.000 and 0.003 to 0.060, respectively (Supplementary Table S3).

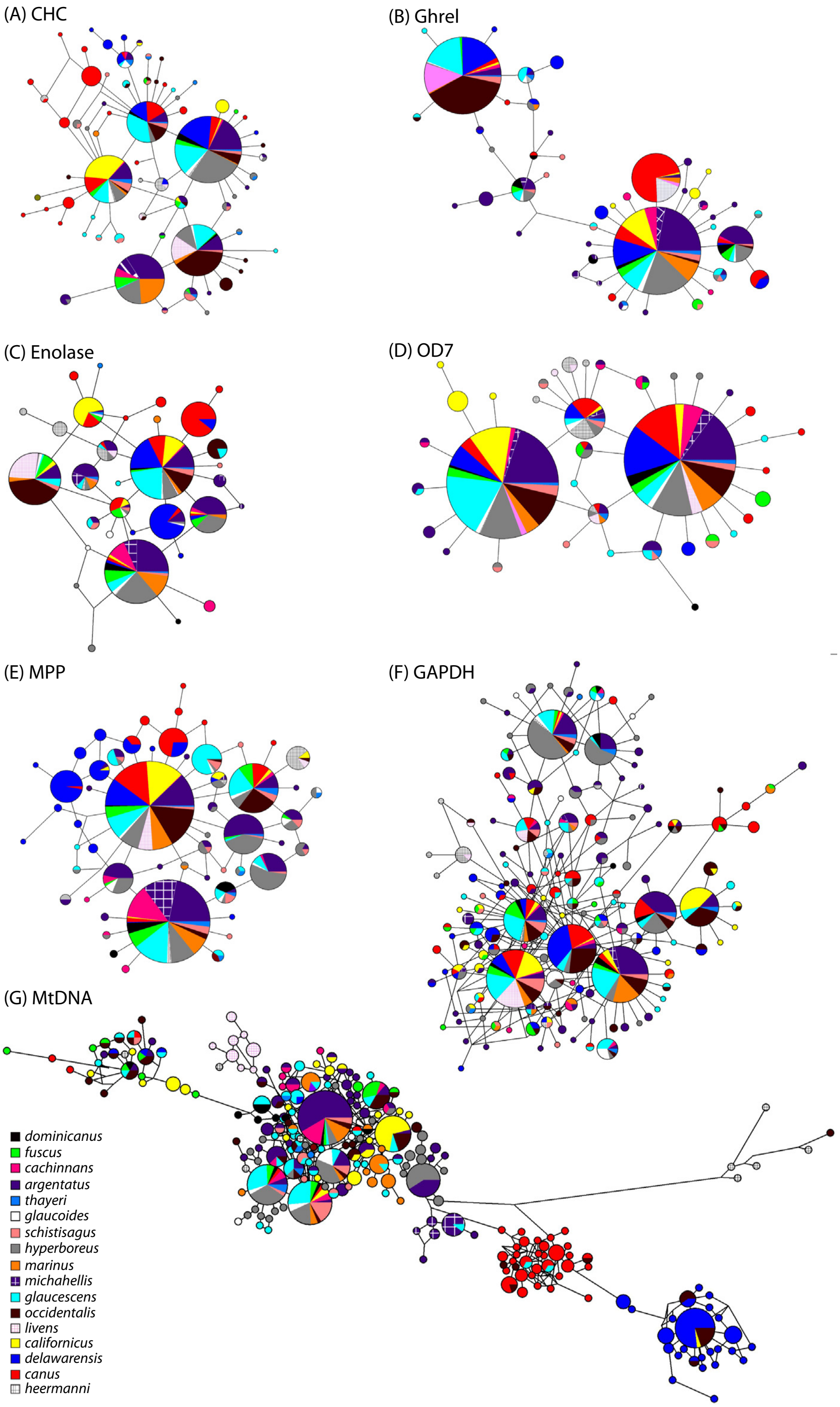

Fig. 2. Parsimony networks illustrating relationships of (A) 31 CHC alleles, (B) 45 Ghrel alleles, (C) 67 enolase alleles, (D) 37 OD7 alleles, (E) 55 MPP alleles, (F) 160 GAPDH alleles, and (G) 235 mtDNA control region haplotypes from *Larus* gulls, with the size of the circle node corresponding to the frequency of each allele. Each sampled species has a unique color.

The DAPC analysis detected genetic structure at both the microsatellite and nuclear intron allelic data sets (Fig. 3). *Canus* and *delawarensis* individuals predominantly formed species-specific clusters based on the microsatellite data (Fig. 3A). Individuals representing *argentatus*, *cachinnans*, *fuscus*, *hyperboreus*, *marinus*, and *michahellis* predominantly clustered together in the lower right quadrant of the DAPC. Individuals representing gulls from western North America (*californius*, *heermanni*, *glaucescens*, *livens*, and *occidentalis*) tended to cluster together in the upper right quadrant of the DAPC. A similar pattern was observed for the DAPC based on the nuclear intron data, except individuals clustered along the PC1 axis, *delawarensis* individuals did not represent a

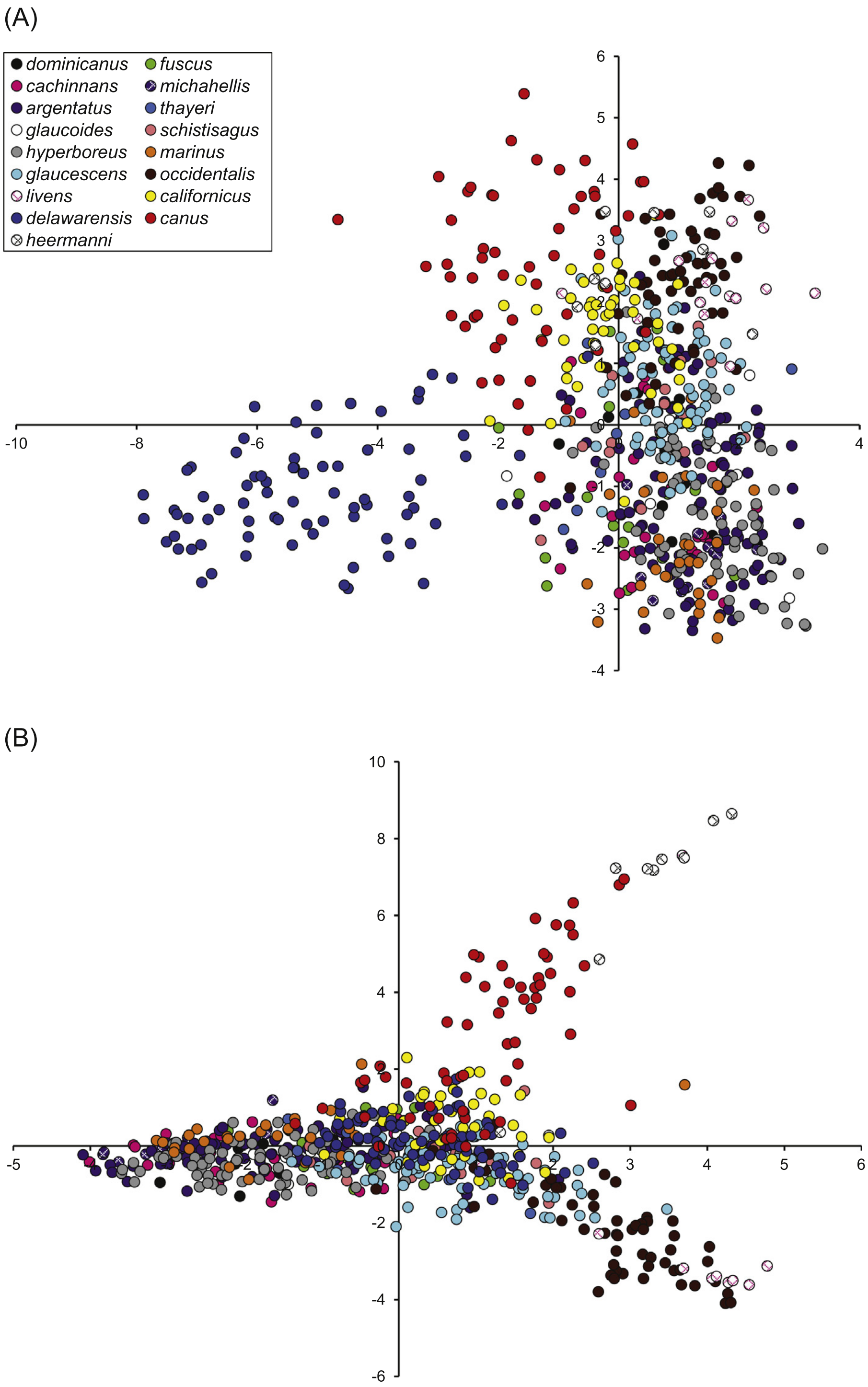

Fig. 3. Scatterplots of the first two principal components of the DAPC from (A) microsatellite and (B) nuclear intron genotype data collected from *Larus* individuals representing 17 species. Each sampled species has a unique color and each dot represents an individual.

distinct cluster, and *canus* and *heermanni* individuals generally formed species-specific clusters (Fig. 3B).

The Bayesian clustering method (STRUCTURE) uncovered genetic partitions within both the microsatellite and nuclear intron allelic data sets (Figs. 4–6). Individuals were placed into two groups identified using Evanno et al.'s (2005) method; *argentatus, cachinnans, dominicanus, fuscus, glaucoides, hyperboreus, marinus, schistisagus*, and *thayeri* (white cluster) and *canus, delawarensis, glaucescens, heermanni, livens*, and *occidentalis* (black cluster). Grouping was concordant between microsatellite and nuclear intron data sets, with a single exception: *californicus* individuals were placed in the white cluster based on microsatellite data (Fig. 4A) and in the black cluster based on allelic intron data (Fig. 4C). Pritchard et al.'s (2000) method identified seven clusters as the optimal number based on the microsatellite data; three clusters were species-specific (*californicus, canus*, and *delawarensis*), whereas the remaining four clusters contained individuals representing multiple species (Fig. 4B). The same method identified four clusters based on the nuclear intron data, all of which contained individuals representing multiple species (Fig. 4D).

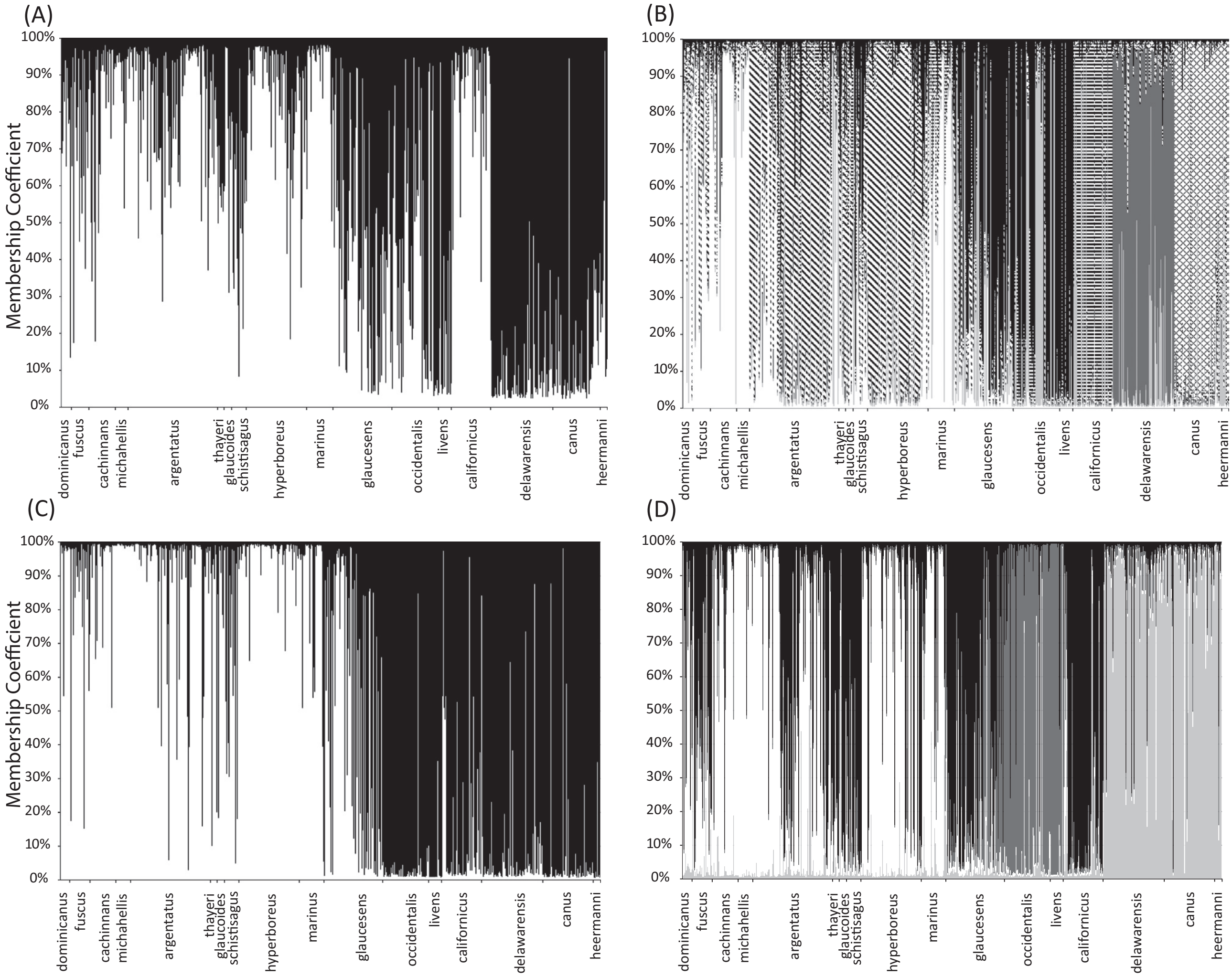

Fig. 4. Structure plots showing assignment of *Larus* individuals representing 17 species (n = 658) into clusters inferred from 11 microsatellite loci following (A) the Evanno et al. (2005) method with two groups and (B) seven clusters based on Pritchard et al. (2000); and inferred from six nuclear intron loci following (C) Evanno et al. (2005) with two clusters and (D) four clusters based on Pritchard et al. (2000).

Subsequent analyses within each of the two clusters identified using Evanno et al.'s (2005) method, uncovered species-based groupings using both microsatellite and intron data (Figs. 5 and 6). Individuals representing *californicus* (Fig. 5A), *canus* (Fig. 6B), *delawarensis* (Fig. 6B), and *heermanni* (Fig. 6B) clustered by species, and New World and Old World subspecies of *canus* (Fig. 6B), based on the microsatellite data. Individuals representing *marinus* and *argentatus argenteus* clustered by species/subspecies and *cachinnans* individuals formed a cluster with *michahellis* individuals when analyzed with European (*argentatus argentatus*, *cachinnans*, and *fuscus*; data not shown) and *dominicanus* gulls. The intron data uncovered similar species-based clusters (Fig. 6C and D), except that individuals representing *marinus* (Fig. 5C) and *glaucescens* (Fig. 6C) clustered by species, albeit weakly. Individuals representing *glaucescens* typically grouped with *schistisagus* individuals based on microsatellite data (Fig. 6A).

3.2. Phylogenetic reconstructions

There is considerable allele and haplotype sharing among white-headed gull species; no locus recovered a species-specific clade (Fig. 2). Among the nuclear loci, alleles representing *canus*, *delawarensis*, and *heermanni* individuals were often placed together in the networks (Fig. 2A–F). More phylogenetic structure was present in mtDNA: haplotypes representing *canus*, *delawarensis*, *heermanni*, *livens*, and *michahellis* individuals formed species clusters, although some haplotype sharing was observed within these groups (Fig. 2G). Interestingly, all 17 species were represented by haplotypes placed in the central cluster (Fig. 2G). The phylogram reconstructed in MrBayes based only on mtDNA yielded a pattern similar to the mtDNA network; the same species clades were observed (although the pp was low for *delawarensis*) and no clade contained individuals representing a single species (Fig. 7). Accordingly, the ML, MP majority rule consensus tree and the consensus tree estimated in MrBayes based on the full data set lacked phylogenetic resolution among white-headed gull species (Fig. 8A). A well supported clade (ML bootstrap support = 100, MP bootstrap support = 99 and 97%; pp = 1) consisting of *heermanni* and a single *occidentalis* individual was basal to the remaining individuals, although representatives of these two species were also present in the large unresolved clade (Fig. 8A). MrBayes recovered three clades containing representatives of a single species or subspecies; *michahellis* (pp = 0.95), *delawarensis* (with a single *occidentalis* representative; pp = 0.99) and *livens* (pp = 0.99; Fig. 8A).

Bayesian analysis of the nuclear and mitochondrial sequence data in BEAST recovered phylogenetic relationships that appeared to place species and subspecies by geographic locality (Fig. 8B). BEAST recovered strong support for a northern European/Icelandic

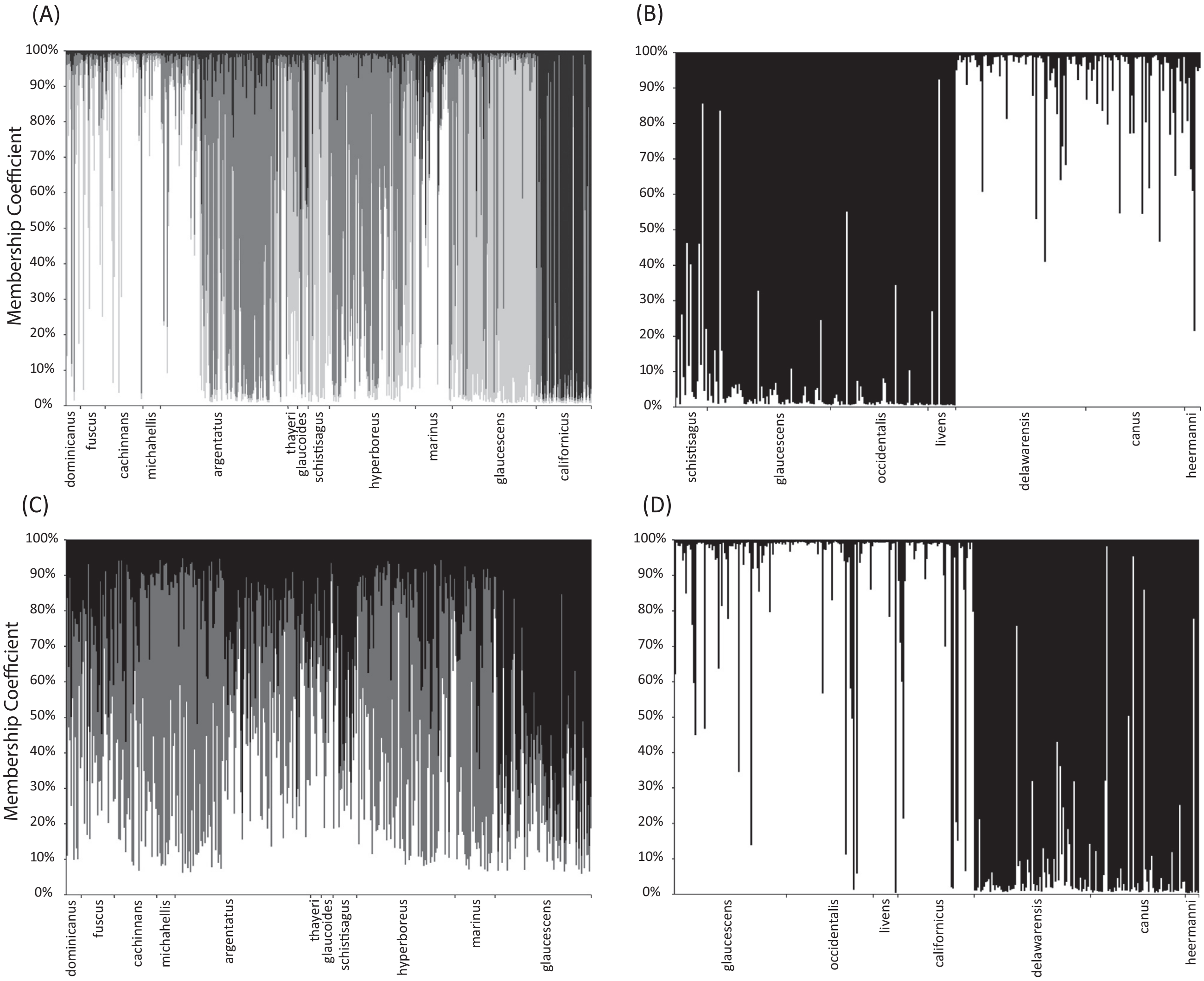

Fig. 5. Structure plots showing assignment of *Larus* individuals within each of the two clusters identified in the global analysis (see Fig. 4A analysis based on microsatellite loci and Fig. 4C analysis based on nuclear intron loci) inferred from 11 microsatellite loci (A and B) and six nuclear intron loci (C and D).

clade (pp = 1.0; *argentatus argentatus*, *argentatus argenteus*, and *hyperboreus* from Norway), a European/Russian clade (pp = 1.0; *argentatus vegae*, *cachinnans*, and *fuscus*), and a western North American/*canus* clade (pp = 0.98; *californicus*, *delawarensis*, *occidentalis*, and *canus*; Fig. 8B). There was weak evidence for a high latitude clade (pp = 0.72) consisting of Scandinavian/Icelandic and northern North American (*argentatus smithsonianus*, *californicus* from Canada, *glaucescens*, *glaucoides*, *hyperboreus* from western Alaska, *hyperboreus* from the Arctic Ocean, *marinus*, and *thayeri*) taxa (Fig. 8B). *Larus canus* subspecies also grouped together with high support (pp = 0.95), as did *argentatus smithsonianus* and *californicus* from Canada (pp = 0.99; Fig. 8B).

4. Discussion

We provide the most comprehensive molecular evaluation of the white-headed gull complex, in terms of geographical coverage, number of species, number of individuals, and marker types, yet conducted. Although our phylogeny was not fully resolved, this multilocus approach resulted in better resolution for relationships of some species than previous studies (Crochet et al., 2000; Pons et al., 2005). Western North American gulls (*heermanni* and *livens*) were sister to the other white-headed gull species: the BEAST analysis placed *canus*, *delawarensis*, and *occidentalis* in a well-supported clade sister to the remaining white-headed gull species (those other than *heermanni* and *livens*), as in phylogenies reconstructed by Crochet et al. (2000) and Pons et al. (2005). In contrast to these previous phylogenies, the BEAST species tree provided some resolution among the remaining white-headed gull species. Specifically, there is strong support for a clade consisting of *cachinnans*, *argentatus vegae*, and *fuscus*, and an *argentatus*–*hyperboreus* clade (see also Liebers et al., 2004), and weaker support for a *californicus*–*argentatus smithsonianus* clade. Phylogenetic trees reconstructed using maximum likelihood, maximum parsimony, and Bayesian inference lacked complete resolution among all white-headed gulls. Lack of phylogenetic signal within genes is likely attributable to the large amount of allele and haplotype sharing, as illustrated in the parsimony networks. When compared with previous studies (Crochet et al., 2003; Liebers et al., 2004; Vigfúsdóttir et al., 2008; Sternkopf et al., 2010) our results show more extensive mitochondrial DNA sharing between species. This discrepancy can be explained in part by our use of shorter mtDNA fragments, allowing less resolution, and in part by the inclusion of a higher proportion of North American individuals, compared with the largely European samples used in previous studies. As shown previously by Sonsthagen et al. (2012) and Pons et al. (2014), there seems to be more extensive mtDNA lineage sharing in North

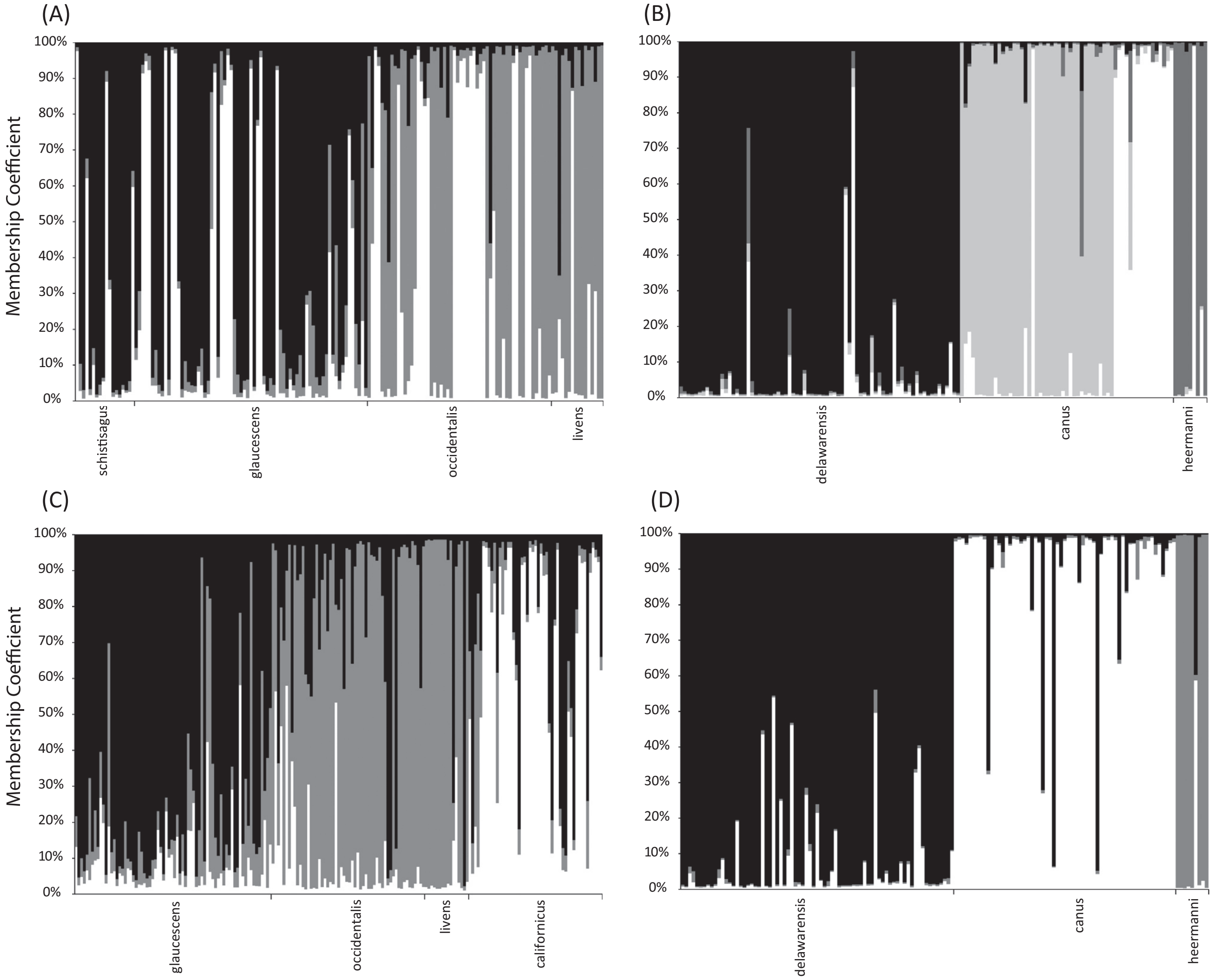

Fig. 6. Structure plots showing assignment of *Larus* individuals within the two clusters identified in the second level analysis (see Fig. 5B analysis based on microsatellite loci and Fig. 5D analysis based on nuclear intron loci) inferred from 11 microsatellite loci (A and B) and six nuclear intron loci (C and D).

America compared with Eurasia. This could be due to the establishment of more recent secondary contact zones in North America and/or multiple glacial refugia in North America, creating many opportunities for species/populations to come into contact and hybridize during periods of glacial contraction (Hewitt, 2001, 2004a, 2004b).

Although microsatellite loci reflect evolutionarily recent events, species clusters were not present in most of the white-headed gull species assayed here. Among gulls with limited phylogenetic resolution, only individuals representing *argentatus argenteus*, *californicus*, and *marinus* grouped by species/subspecies. Conversely, individuals representing *livens*–*occidentalis* (Fig. 6A), *glaucescens*–*schistisagus* (Fig. 6A), *argentatus smithsonianus*–*hyperboreus* (Fig. 5A), and *michahellis*–*cachinnans* (data not shown) grouped together, indicating that introgression and retention of ancestral polymorphism may be obscuring evolutionary relationships. It is important to note that the taxonomy of this group is controversial (e.g., *argentatus*, *smithsonianus* and *mongolicus* are considered valid species by some taxonomic references; see Gill and Donsker, 2016). Therefore, some of the cases of para/polyphyly at the species level in our study might be attributable to imperfect systematics.

Introgression of mtDNA haplotypes was pervasive, as no species-specific haplotype clades were observed (Fig. 2G). This is of particular interest because introgressed haplotypes were observed between species pairs where reported hybridization is infrequent (e.g. *delawarensis* × *californicus*; McCarthy, 2006) and between sympatric species with no confirmed records of hybridization (e.g. *canus* × *glaucescens*; *delawarensis* × *occidentalis*). The observation of introgressed haplotypes between species with no confirmed accounts of hybridization may be the result of recent hybridization events, retention of introgressed haplotypes from historical hybridization events, or indirect transfer via mating with an individual that retains a non-species specific haplotype from a previous hybridization event. Because hybrid individuals do not appear to experience reduced fitness in cases documented to date (Good et al., 2000; Neubauer et al., 2009), introgressed haplotypes would likely not be eliminated from populations. Incomplete lineage sorting may also explain the observed pattern shared haplotypes, specifically shared haplotypes located centrally in the network (Fig. 2G). However, it is unlikely to explain all instances of haplotype sharing observed among diverged lineages noted above. Alternatively, observations of introgression of haplotypic data could be the result of laboratory artifacts. However, samples with introgressed haplotypes were included in our quality control procedures: these samples were either re-extracted and re-sequenced from this independent extraction, or re-amplified

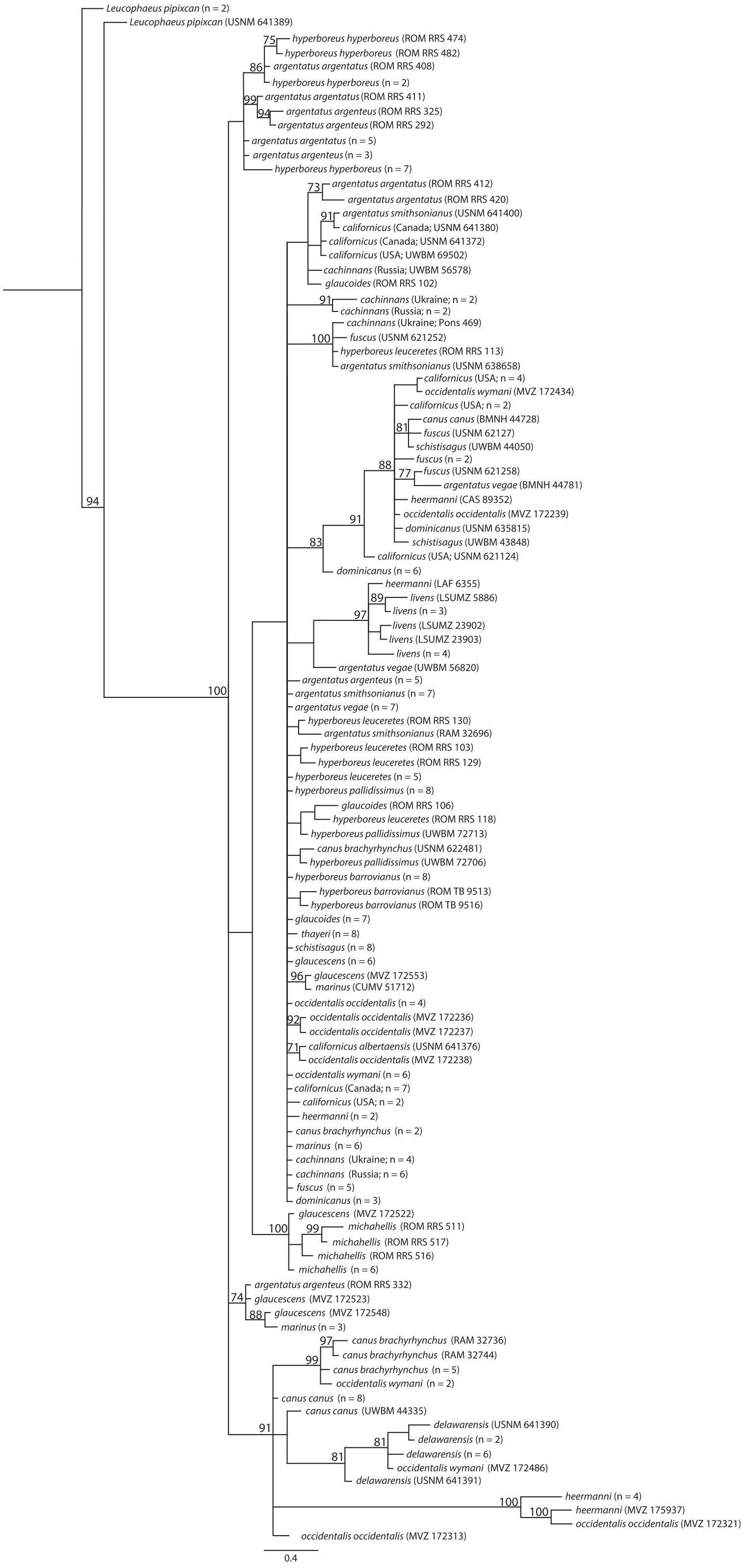

Fig. 7. Phylogeny of *Larus* gulls inferred from mtDNA control-region sequences. The number of individuals represented at each branch is in parenthesis, except for single individuals, in which case individual reference numbers are provided. Support values at nodes are Bayesian posterior probabilities from MrBayes. Ten individuals per taxon were included in the analysis.

and re-sequenced from the original extractions. We detected no inconsistencies between replicates. We thus feel confident that we can exclude experimental error as a general explanation for the high level of haplotype sharing between these strongly differentiated species. Further investigation of the breeding behavior between these species pairs is warranted to distinguish among historical and contemporary hybridization and incomplete lineage sorting.

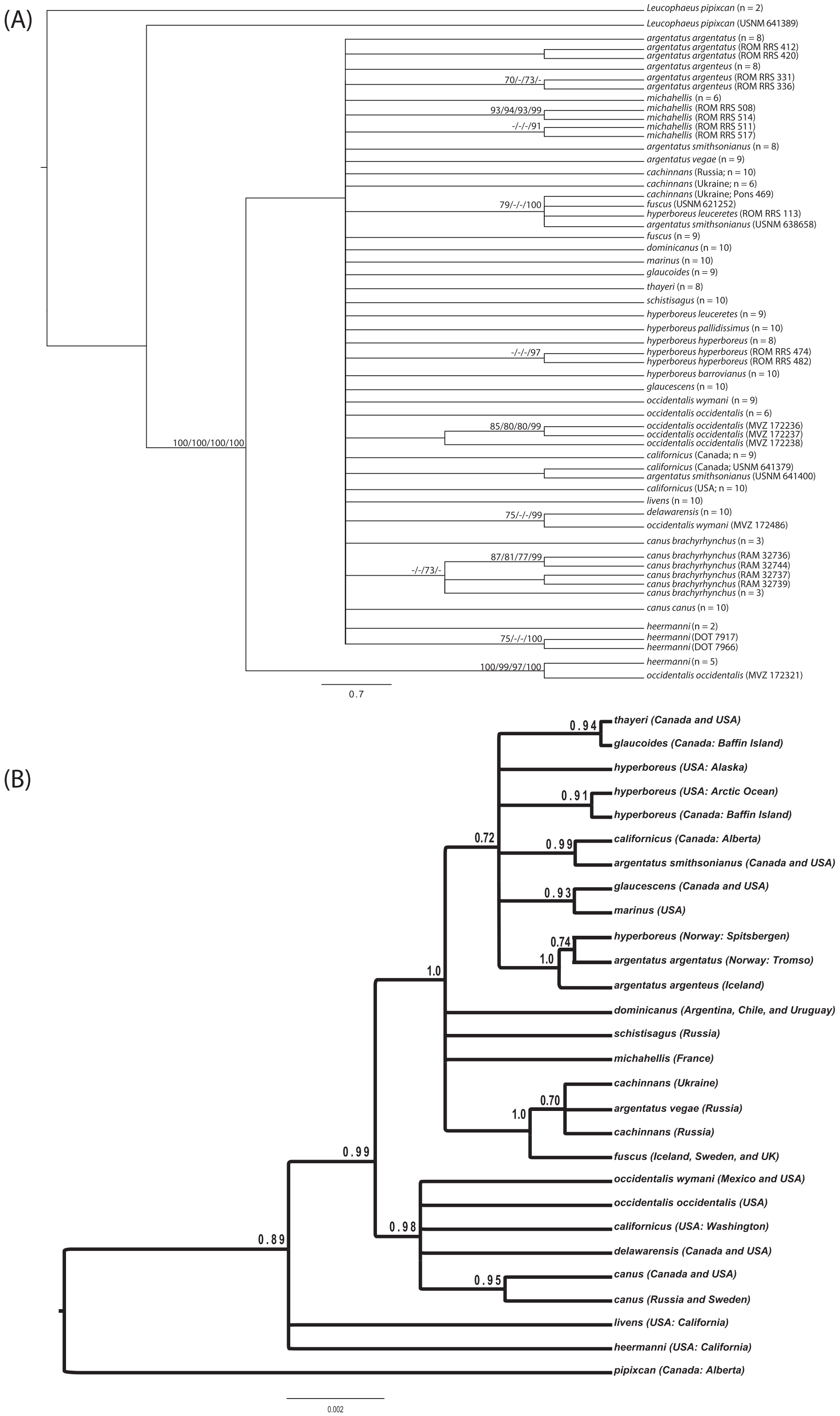

Fig. 8. Phylogenetic reconstructions of *Larus* gulls, based on sequences of the mtDNA control-region and six nuclear intron loci, and estimated using (A) maximum likelihood (ML) in RaxML, maximum parsimony (MP) in PAUP (topology shown) and Bayesian approaches in MrBayes and (B) BEAST. Numbers at nodes in A represent (1) ML support, (2) MP support without and (3) with treating insertion/deletions as a fifth state, and (4) Bayesian posterior probabilities; numbers at nodes in B are Bayesian posterior probabilities. Bootstrap support values and posterior probabilities <0.70 are denoted by a dash. The number of individuals represented at each branch in A is in parentheses, except for single individuals, in which case individual reference numbers are provided in parentheses. Ten individuals per taxon were included in the analyses.

Recent evolution of species in the white-headed gull complex is not a sufficient explanation for the limited phylogenetic resolution within this group. If recent evolutionary history were the primary factor limiting phylogenetic reconstruction, analyses of the rapidly mutating microsatellite loci would presumably have identified more species-specific clusters within the highly-related clade than did analysis of the nuclear intron data (cf. Sonsthagen et al., 2011). Recurrent gene flow among sister species will act as a homogenizing force, increasing the probability of reconstructing the correct species tree by decreasing the observed differences among sister taxa but increasing the evidence for shared ancestry (Leaché et al., 2014). Among non-sister taxa (paraphyletic), however, gene flow generates discordant gene trees that further obscure species relationships. Ecological and sexual selection likely play a central role in the divergence of morphological and behavioral characteristics in allopatric and parapatric populations (Rasner et al., 2004; Yeh, 2004). Mechanisms to maintain species barriers after secondary contact dictate whether changes that originated in isolation are sufficient to complete the speciation process (Edwards et al., 2005) or result in the merging of species (e.g., despeciation; Webb et al., 2011). Development of pre-zygotic barriers to reproduction is especially important within birds, as avian species typically retain hybrid viability and fertility for millions of years after speciation (Price and Bouvier, 2002). As evident by the limited phylogenetic signal (MP, ML, MrBayes, BEAST), mixed species clusters (STRUCTURE), and frequent hybridization among members of the white-headed gull complex, ecological and sexual selection have not been strong enough during the time since they became separated to complete reproductive isolation within this recent species complex.

Among recently evolved species, the divergence of characters involved in mate choice is likely central to maintaining species limits (Edwards et al., 2005). In areas of sympatry, however, the discrimination of traits (i.e., female preference) is likely more important than the evolution of traits themselves (Hudson and Price, 2014). There is evidence of mate recognition between some species pairs (e.g., *argentatus–cachinnans*; Neubauer et al., 2009), and of stable hybrid zones between some species (e.g., *glaucescens–occidentalis*; Good et al., 2000). Likewise, traits known to be involved in mate choice in gulls exhibit sharper clines and reduced introgression in two hybrid zones (Gay et al., 2007, 2008), and nuclear introgression has been limited between some species pairs (e.g. *argentatus smithsonianus–marinus*; Pons et al., 2014). However, these may represent exceptions in this complex, especially among North American forms where hybridization is more extensive. Within members of the white-headed gull complex, it appears that their "windows of recognition" are broad (*sensu* Hudson and Price, 2014) and the lack of reinforcement and strong post-mating isolation does not limit hybridization. Indeed, assortative mating is not complete and introgression is often pervasive in areas of secondary contact. Individuals occupying high latitudes, notably in North America, tended to cluster by locality rather than species (e.g. *argentatus smithsonianus* and *hyperboreus*, Sternkopf et al., 2010; Sonsthagen et al., 2012), and even within the BEAST species tree, species appear to group by geography, a pattern best explained by recurrent hybridization among sympatric species (Fig. 6B). Moreover, when species do come into secondary contact and hybridize, the resulting offspring do not appear to suffer from loss of fitness (Good et al., 2000; Neubauer et al., 2009; but see Neubauer et al., 2014). Indeed, species/populations having generalist requirements, as do many species of white-headed gulls, and living in similar habitats are less likely to be reproductively isolated (e.g., Nosil et al., 2009).

Hybridization is widespread in birds (9.2% of all species hybridize), with incidence of hybridization in 41.6% of species within some orders (i.e. Anseriformes; Grant and Grant, 1992). Natural hybridization appears to be a widespread feature of the evolutionary history of several avian species complexes (Grant and Grant, 1992; Kraus et al., 2012) and may lead to speciation in some cases (Hermansen et al., 2014). Although hybridization breaks epistatic interactions between gene complexes (Coyne and Orr, 2004; Kopp and Frank, 2005), recently diverged species likely have had insufficient time to evolve genetic incompatibilities with closely related species. In these instances, hybridization may be beneficial by increasing genetic diversity and may even result in hybrid superiority (see Grant and Grant, 1992). The recent evolutionary history and propensity to hybridize among white-headed gulls complicate the ability to resolve species relationships. Hybridization does appear to be a recurrent evolutionary force within at least some species of white-headed gulls, notably the North American species/subspecies. If hybridization were limited to a single nuclear introgression event, the genetic legacy of the event would be lost within six generations (Lavretsky et al., in press). As noted with other species, perhaps just a few genes are driving the speciation process within this complex (Poelstra et al., 2014; Mason and Taylor, 2015). Alternatively, species divergences could be determined by a large number of small-effect loci maintained in linkage disequilibrium by divergent selection (Kremer and Le Core, 2012; though see Savolainen et al., 2013; Yeaman, 2015). Therefore, examination of functional genes associated with morphological characteristics may shed further light on interspecific relationships and speciation in the white-headed gull complex.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.06.008.

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