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Working through polytomies: Auklets revisited

Elizabeth M. Humphries *, Kevin Winker

University of Alaska Museum, Department of Biology & Wildlife, Institute of Arctic Biology, 907 Yukon Drive, Fairbanks, AK 99775, USA

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ABSTRACT

Polytomies, or phylogenetic "bushes", are the result of a series of internodes occurring in a short period of evolutionary time (which can result in data that do not contain enough information), or data that have too much homoplasy to resolve a bifurcating branching pattern. In this study we used the *Aethia* auklet polytomy to explore the effectiveness of different methods for resolving polytomies: mitochondrial DNA gene choice, number of individuals per species sampled, model of molecular evolution, and AFLP loci. We recovered a fully-resolved phylogeny using NADH dehydrogenase subunit 2 (ND2) sequence data under two different Bayesian models. We were able to corroborate this tree under one model with an expanded mtDNA dataset. Effectiveness of additional intraspecific sampling varied with node, and fully 20% of the subsampled datasets failed to return a congruent phylogeny when we sampled only one or two individuals per species. We did not recover a resolved phylogeny using AFLP data. Conflict in the AFLP dataset showed that nearly all possible relationships were supported at low levels of confidence, suggesting that either AFLPs are not useful at the genetic depth of the *Aethia* auklet radiation (7–9% divergent in the mtDNA ND2 gene), perhaps resulting in too much homoplasy, or that the *Aethia* auklets have experienced incomplete lineage sorting at many nuclear loci.

1. Introduction

The goal of most phylogenetic studies is to infer a completely resolved, bifurcating phylogeny. The presence of polytomies, or phylogenetic "bushes", in the final phylogeny is often seen as a failure (Rokas and Carroll, 2006). These polytomies may be artifacts of the inference process—"soft"—or may be biologically real—"hard" (Maddison, 1989). Theoretically, soft polytomies can be resolved if one corrects the problems in the inference process, although this can be difficult if the amount of time since the radiation (length of the terminal branches) is long compared to the amount of time between speciation events (length of the internode; Rokas and Carroll, 2006; Whitfield and Lockhart, 2008). The stochastic process of lineage sorting becomes increasingly problematic as internode lengths shrink; different genes will infer different relationships, or several different relationships will be supported by different parts of the same gene (Whitfield and Lockhart, 2008). As the internodes shrink relative to the terminal branches, soft polytomies become harder to resolve, eventually turning into hard polytomies (Walsh et al., 1999). Thus, polytomies exist on a continuum of resolvability. Hard polytomies, by definition, cannot be resolved into bifurcating relationships.

Different methods have been proposed for determining whether a polytomy is soft or hard, and many of these are tree-based. For example, Slowinski (2001) advocated a method that involved topology testing, which Poe and Chubb (2004) expanded by suggesting that researchers should determine whether multiple independent gene trees are congruent, as opposed to simply testing whether a resolved topology better fits the data than a polytomy. McCracken and Sorenson (2005) also used a multiple-locus approach to resolving polytomies and included both parametric and nonparametric bootstrapping. Recently, several studies have used a coalescence-based approach to infer a species tree from multiple gene trees that have experienced incomplete lineage sorting (Carstens and Knowles, 2007; Liu and Pearl, 2007; Maddison and Knowles, 2006). All of these methods used sequence data. DNA sequencing is a useful phylogenetic tool, as evidenced by its popularity in systematic studies; and it has many advantages, including repeatability and well-characterized models of evolution (Avise, 2004). However, analyses using sequence data can be hampered by choice of gene and by limited genomic representation. Practically, sequence-based studies can only examine a very small fraction of the total genome and, thus, of the total phylogenetic information that the genome contains. Fragment-based methods, such as amplified fragment length polymorphisms (AFLPs; Vos et al., 1995), may be able to compensate for this particular weakness of sequence-based methods. There are several benefits to the AFLP method: (1) it involves a sampling of the entire genome, resulting in a broad assessment of genomic phylogenetic signal; (2) it samples many (presumably) unlinked loci; and (3) many of the loci sampled are polymorphic (often more than 50%, depending on how closely taxa are related), which may result in greater phylogenetic signal (e.g., Ribiero et al., 2002; Schneider et al., 2002).

* Corresponding author. Fax: +1 907 474 5469.
E-mail address: ftemh@uaf.edu (E.M. Humphries).

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Koopman (2005) suggested that AFLPs have more phylogenetic signal at lower divergence levels than ITS sequence data in fungi, although this relationship was not clearly seen in plants or bacteria. Spooner et al. (2005) suggested that AFLPs were more useful than sequence data in resolving relationships among wild tomatoes. AFLPs have become widely used in plants but are relatively uncommon in vertebrate studies (Bensch and Åkesson, 2005).

Increasing the number of individuals per species sampled may also increase phylogenetic resolution. Many studies have examined whether choice of different ingroup taxa sampled is more important for inferring resolved phylogenies than increasing the number of loci sampled; most suggest that a larger number of loci results in greater phylogenetic resolution (Rosenberg and Kumar, 2003; Rokas et al., 2005; but see also Hedtke et al., 2006). Additionally, Erdös et al. (1999) suggested that increasing the length of the sequences does not necessarily improve phylogenetic inference. Jackman et al. (1999) suggested analyzing subsampled datasets with fewer taxa to resolve polytomies. However, examining only one individual per species potentially overlooks phylogenetically informative intraspecific variation and could effectively mean ignoring sampling error and incorporating sampling bias (based on which individual is chosen) into the dataset (Ives et al., 2007).

One basic assumption in resolving soft polytomies is that repeating analyses with larger and larger datasets will result in a greater resolution until the polytomy disappears (Maddison, 1989; DeSalle et al., 1994). However, because of systematic bias (such as long branches rich with homoplasy), phylogenetic analysis of large datasets can produce fully resolved and well supported but erroneous topologies (Rokas and Carroll, 2006). Thus, increasing the size of a dataset may not be as informative as an approach that can examine phylogenetic signal in the data independent of a single topology (Charleston and Page, 1999). Network methods, such as spectral analysis (Hendy and Penny, 1993), NeighborNet (Bryant and Moulton, 2004) and consensus networks (Holland and Moulton, 2003; Holland et al., 2005, 2006) allow visualization of all possible phylogenetic relationships in the data, not just the majority or plurality relationship (Kennedy et al., 2005). In particular, consensus networks show all bifurcations that appear in a given input of trees (Kennedy et al., 2005), permitting one to examine more than just the consensus tree in likelihood or Bayesian phylogenetic analyses.

In this study, we examined the utility of increased sampling (genomic and individual) and improved evolutionary models and analytical methods to re-examine the polytomy described by Walsh et al. (1999) in *Aethia* auklets (Aves: Alcidae). Auklets are small seabirds that breed on rocky shorelines and spend the rest of the year at sea (Byrd and Williams, 1993; Jones, 1993a,b; Manuwal and Thoresen, 1993; Jones et al., 2001). Auklets belong to three genera: (1) *Cerorhinca*, a monotypic genus consisting of *C. monocerata* (rhinoceros auklet, a widely acknowledged distant relative to the group of interest here); (2) *Ptychoramphus*, another monotypic genus (*P. aleuticus*, Cassin's auklet); and (3) *Aethia*, a genus with four members: *A. pusilla* (least auklet), *A. psittacula* (parakeet auklet), *A. cristatella* (crested auklet), and *A. pygmaea* (whiskered auklet). The earliest molecular and morphological phylogenies of the Alcidae placed *Ptychoramphus* as sister to a polytomy formed by all the members of *Aethia* (Strauch, 1985; Moum et al., 1994; Friesen et al., 1996). However, despite repeated study, researchers have been unable to resolve the relationships within *Aethia* (Moum et al., 1994; Friesen et al., 1996; Walsh et al., 1999; Pereira and Baker, 2008).

Walsh et al. (1999) speculated that the *Aethia* auklets diverged ~2.6 million years ago and that their speciation may have been the result of late Pliocene/early Pleistocene glaciations. They used a power-analysis approach and determined that if the *Aethia* auklets had speciated over 100,000 years (the range of the shortest interglacial period in the late Pliocene/early Pleistocene), enough DNA (~3000 base pairs) had been sequenced in their study to resolve the *Aethia* polytomy if it was soft. Additionally, they discovered that the number of base pairs required to resolve a polytomy increases exponentially (Walsh et al., 1999). Thus, they estimated that ~22,000 base pairs of equivalently informative mtDNA would be required to resolve the polytomy if the range of radiation decreased by a power of 10 (i.e., to about 10,000 years). Because the avian mtDNA genome only has ~17,000 base pairs (Mindell et al., 1999), this polytomy was considered essentially unresolvable by using mtDNA sequence data (assuming the radiation occurred over 10,000 years or less). Pereira and Baker (2008) combined sequences from five mitochondrial and one nuclear gene; they suggested that the unresolved *Aethia* relationships are the result of incomplete lineage sorting. Thus, the *Aethia* polytomy would remain no matter how much data were added.

The presence of an apparently hard polytomy in the auklets, coupled with a radiation presumed to have occurred over less than 100,000 years, make *Aethia* an ideal genus for testing various methods for resolving polytomies. We employed three strategies to crack the *Aethia* polytomy: increasing genomic data, sampling multiple individuals within each species, and running more sophisticated analyses. First, we tested Walsh et al.'s (1999) conclusion that the auklet polytomy is "hard" using a different mtDNA gene (NADH dehydrogenase 2, ND2), amplified fragment length polymorphisms (AFLPs), and a Bayesian mixture model of nucleotide substitution. We also combined our data with the sequence data from Pereira and Baker (2008). We then examined how phylogenetic signal and support in the data changed as we increased the number of individuals per species. Finally, we used a coalescent method to estimate the duration of the *Aethia* radiation.

2. Methods

2.1. Mitochondrial DNA (mtDNA) sequencing

DNA was extracted from muscle tissue from 9 *Ptychoramphus*, 10 *A. psittacula*, 10 *A. pusilla*, 10 *A. pygmaea*, and 10 *A. cristatella* individuals (Table 1) using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). We amplified the mtDNA NADH dehydrogenase subunit 2 (ND2) gene using primers L5215 (Hackett, 1996) and H6313 (Sorenson et al., 1999). Amplification was done following standard PCR protocols and using an annealing temperature of 48 °C. Cycle-sequencing was done using ABI Big-Dye Terminator mix and an annealing temperature of 50 °C. Products were sequenced on an ABI 3130 automated sequencer (Applied Biosystems, Inc., USA). We aligned sequences without gaps using Sequencher (Genecodes Corporation, Inc., USA).

2.2. Amplified fragment length polymorphism (AFLP) analyses

We chose five individuals from each species for AFLP analysis (Table 1). We followed a modified ABI (Applied Biosystems Inc., USA) plant-mapping (large genome) protocol to generate loci (Parchman et al., 2006). Restriction of extracted DNA using enzymes MseI and EcoRI was done concurrently with ligation of MseI and EcoRI adaptors. Preselective amplification was done using ABI's preselective primers. We used 11 primer pair combinations for selective amplification (Appendix 1). Genotyping was done on an ABI 3100 automated sequencer (Applied Biosystems Inc., USA). We scored the chromatograms using GeneMapper ver. 3.7 (Applied Biosystems, Inc., USA). Only loci that could be determined unambiguously as present or absent in each individual (i.e., clean, well-defined peaks) were scored. We used a minimum peak width of 1.5 base pairs and a minimum peak height of 100 as a starting

Table 1
Individuals used in this study. Collecting localities (all from Alaska), and identifiers (specimen voucher numbers) are included. All specimens (except *Aethia cristatella*) are deposited in the University of Alaska Museum. All individuals were used for sequencing analysis; starred individuals were used for AFLP analysis.

Species	Voucher No.	Collection localities	GenBank Accession Numbers
Ptychoramphus aleuticus	UAM 13178, 11284*, 14171*, 14557*, 20537*, 23581, 23582, 23583, 23723, UAMX 2982*[a]	Buldir Is., Chowiet Is., Isl. of Four Mts, Little Koniuji Is., Lowrie Is. (5).	GU014413 - GU014421
Aethia cristatella	UAMX 4529*, UAMX 4530*, UAMX 4531*, UAMX 4532*, UAMX 4533, UAMX 4535*, UAMX 4539, UAMX 4541, UAMX 4540, UAMX 4542	St. Lawrence Is. (10)	GU014422 - GU014431
Aethia psittacula	UAM 10191, UAM 10192*, UAM 20539*, UAM 20540, UAM 22045*, UAM 22464, UAM 22465, UAM 23721, UAM 23747*, UAMX 4400*	Koniuji Is. (5), St. George Is., St. Paul Is. (4)	GU014432 - GU014441
Aethia pusilla	UAM 18490, UAM 20141*, UAM 20158*, UAM 20201*, UAM 20210, UAM 20211*, UAM 23742, UAM 23745, UAM 23746, KSW 4727*	Birch Creek, Buldir Is., Little Diomede Is., St. George Is. (6), St. Paul Is.	GU014442 - GU014451
Aethia pygmaea	UAM 8851*, UAM 8853*, UAM 8856*, UAM 9877, UAM 9978*, UAM 14841, UAM 14843, UAM 14844, UAM 14846, UAM 23722*	Konuiji Is., Sedanka Is. (4), Seguam Is., Umak Is. (4)	GU014403 - GU014413

[a] Specimen used for AFLP analysis.

point, but then examined each peak individually to maximize the amount of phylogenetic information obtained (Holland et al., 2008).

2.3. Phylogenetic analyses

2.3.1. mtDNA

We used MrModelTest (ver. 2.2) by Nylander (2004) in combination with PAUP* (Swofford, 2002) to choose the most appropriate model (GTR + I + Γ) for phylogenetic analysis. We chose the model based on the AIC criterion. In addition, we also ran analyses under the GTR + site-specific (SS) model, in which the data were partitioned by codon site. Under this model there is no gamma distribution or invariant sites assumption; instead, the overall rate of mutation varies among partitions (all other parameters remained the same for each position). (The GTR + SS model was 200 log-likelihood units better than the GTR + I + Γ model.) Phylogenetic analyses were done using MrBayes (ver. 3.1.1, Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Analyses were run using four chains and flat Dirichlet priors. Both analyses were run for 8 million generations with a burnin of 3 million generations and a tree sampling interval of 10,000, for a total of 501 trees sampled. The length of the burnin period for this run and all subsequent MrBayes runs was chosen based on when the differences between the −ln of the likehoods for the cold chain and one randomly-chosen heated chain were consistently less than 0.1.

We also used the sequence data of Pereira and Baker (2008) for the five auklet species (Appendix 2). We downloaded a total of 43 sequences, a combination of five mitochondrial genes, one nuclear gene (recombination activating gene 1 [RAG-1]), and 18 individuals: 5 individuals each for cytochrome *b*, NADH dehydrogenase subunit 2 (ND2), 16S and 12S, and 18 individuals for cytochrome *c* oxidase subunit I (COI). We only included sequence data with an associated vouchered specimen. We concatenated all mitochondrial data from the same individual; there were only five individuals (one *Ptychoramphus* and one of each of the four *Aethia* species) with sequences for all five mitochondrial genes. We then aligned the remaining downloaded mitochondrial sequences (all for COI) with the COI genes in the concatenated dataset and coded the remaining genes as missing data. We repeated this for our ND2 dataset. In the end, we had a data matrix containing 68 individuals and 4744 bp of mtDNA (although data were missing for some individuals). We also created a second mitochondrial matrix by combining our GenBank dataset with only three individuals per species from our ND2 dataset. (We chose to include three individuals because on average that is the number of additional sequences we downloaded from GenBank for COI.) We chose to concatenate the data because the genes were all from the mitochondrial genome and thus share a similar evolutionary history; we also chose to include individuals for which we did not have all genes sequenced, because even datasets with missing data can infer accurate phylogenies (Wiens et al., 2005). We analyzed the RAG-1 dataset separately because genes from the nuclear genome do not necessarily share the same history as the mitochondrial genome. We partitioned both mitochondrial matrices by gene and used MrModelTest (ver. 2.2; Nylander, 2004) in conjunction with PAUP* (Swofford, 2002) to determine the most appropriate model for each gene. We also used this method to determine the best model for the RAG-1 dataset. In all cases, the GTR + I + Γ model was found to be the most appropriate model. However, the GTR + SS model (which is not a model tested by MrModelTest) was over 200 log-likelihood units better than the GTR + I + Γ model (calculated using PAUP*). We used the same priors as before, but ran analyses for the combined mitochondrial datasets for 12 million generations, sampling trees every 10,000 generations. We used a burnin of 5 million generations and sampled a total of 701 trees. We then repeated analysis of both mitochondrial matrices using the GTR + SS model and the same priors and settings. The RAG-1 dataset was analyzed using both models and run for 8 million generations, with a burnin of 3 million generations and trees sampled every 10,000 generations.

To determine the effect of increased sample size on polytomy resolution, we randomly subsampled the ND2 dataset without replacement and created phylogenies using 1–9 individuals per species. We replicated this five times. These phylogenies were created using the GTR + SS models with the same settings as the full ND2 dataset and were reconstructed to answer two questions: did the analysis of the subsampled dataset result in the same phylogeny as the full dataset; and what was the posterior probability of the internal nodes?

2.3.2. AFLPs

One of the limitations of the AFLP method for phylogenetic studies is the lack of a satisfactory model of AFLP evolution. Most researchers use parsimony for AFLP analysis. Because parsimony has been shown to fail in some cases (Felsenstein, 2004), or to falsely resolve a real polytomy (Slowinski, 2001), using this method for phylogenetic analysis could result in incorrect inference. We inferred phylogenies using both parsimony and Bayesian methods. Parsimony analysis was performed in PAUP* (Swofford, 2002). The Bayesian analysis was done in MrBayes (ver. 3.1.1, Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) using the restriction site (binary) model. These analyses were run using four chains for 8 million generations with a burnin of 3 million generations and a tree sampling interval of 10,000. To examine the conflict in the dataset, we used the bipartition probabilities file

from MrBayes in SplitsTree4 (ver 4.6; Huson and Bryant, 2006). We only examined bipartitions that were present in greater than 5% of trees.

All trees from mtDNA sequence data and AFLP data were rooted with *Ptychoramphus*, a monotypic genus known to be basal to the *Aethia* polytomy (Strauch, 1985; Friesen et al., 1996).

2.4. Estimation of the length of the Aethia radiation

We used the program BEAST (Drummond and Rambaut, 2007) to estimate the time to most recent common ancestor (TMRCA) at each node and the duration of the *Aethia* radiation. TMRCA estimates were made in standard time units (STU), which are the estimated number of substitutions per 1041 base pairs (the length of the ND2 gene) along a branch. We downloaded ND2 sequences for *Fratercula arctica*, *Cerorhinca monocerata*, *Cepphus columba*, *Uria lomvia*, *Alca torda*, *Alle alle*, *Brachyramphus brevirostris*, and *Synthliboramphus antiquus* from GenBank (Accession Nos. DQ385092, EF373230, EF373229, EF373273, EF373220, EF373221, EF373227, and EF373269, respectively), and subsampled our ND2 dataset, randomly choosing three different individuals per *Aethia* species. TMRCA was estimated using an HKY + I + Γ model under a strict molecular clock with a mean substitution rate of 1.0. (We were unable to get reasonable posterior distributions for all the parameters when using a more complex model.) We used a Yule process tree prior and estimated our starting tree using UPGMA. We ran BEAST for 14 million generations, sampling every 10,000 generations and using a burnin of 10%.

3. Results

3.1. Aethia phylogeny

One thousand forty-one base pairs of mtDNA were generated for 49 individuals (Table 1). We generated a single consensus tree (using the 50% majority rule) for each Bayesian run using only our ND2 dataset. The genus *Aethia* was recovered as monophyletic with a posterior probability of 1.0 under both the GTR + I + Γ and the GTR + SS models. Additionally, each species was monophyletic, as expected, and trees from both models of molecular evolution showed the same topology: *A. cristatella* and *A. pygmaea* were recovered as sister taxa, *A. psittacula* was sister to the clade containing *A. cristatella* and *A. pygmaea*, and *A. pusilla* was recovered as basal to the rest of the *Aethia* auklets (Fig. 1). The same topology was recovered when we ran the analyses under a strict clock.

We also generated 898 AFLP loci for a total of 25 individuals using 11 different primer pair combinations (average 81.6 loci per primer pair with a standard deviation of 15.4 loci). Loci were 75–300 bp in size. This dataset proved to have little strength to resolve relationships in this group. Parsimony failed to resolve the

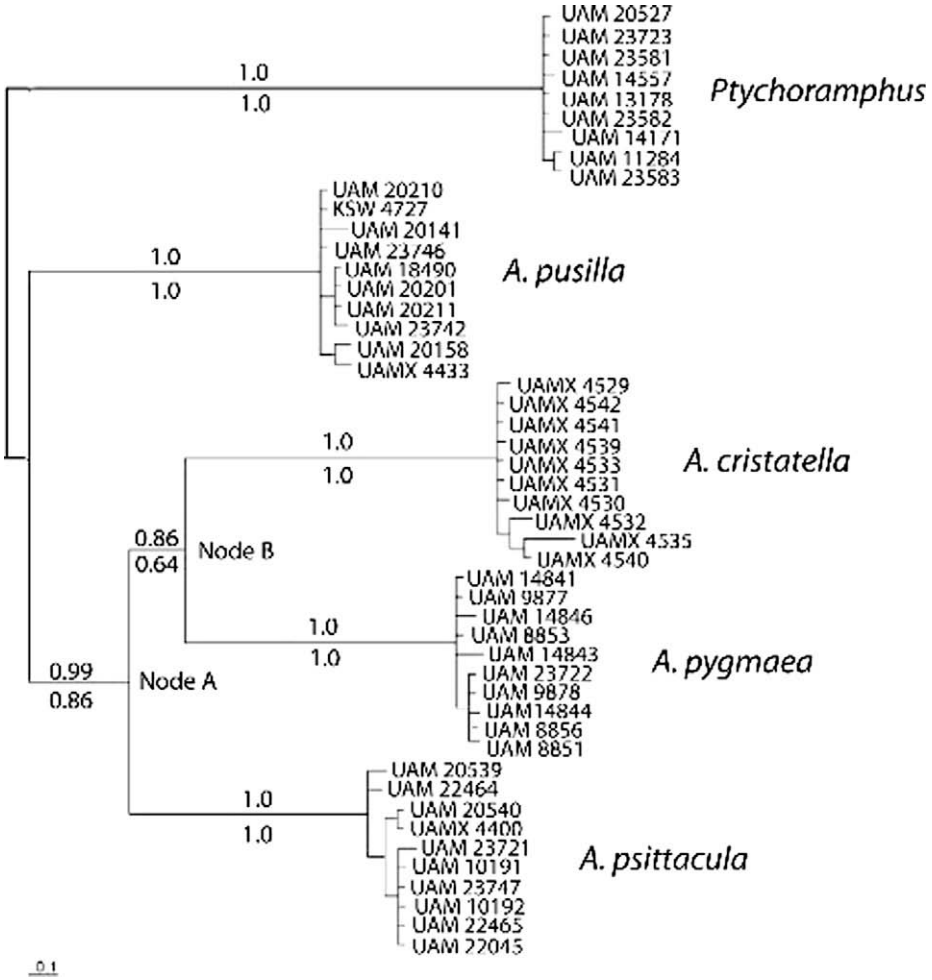

Fig. 1. Bayesian mtDNA phylogeny (ND2 data). The same topology was recovered under both models of evolution. The number on top of the nodal branches is the posterior probability under the GTR + I + Γ model of evolution; the number below the branches is the posterior probability under the GTR + SS model. The two nodes tested for sample size effects are labeled A and B.

polytomy, and Bayesian analysis produced a consensus tree that recovered *A. cristatella* and *A. pusilla* as sister with a posterior probability of only 0.55, with *A. pygmaea* as sister to the clade that contains *A. cristatella* and *A. pusilla* (Fig. 2). *A. psittacula* was recovered as basal to the rest of *Aethia*. All species were recovered as monophyletic with a posterior probability of 1.0. When we examined the conflict in the dataset (i.e., other possible bipartitions recovered during Bayesian analysis), we discovered that most possible sister relationships among the auklets were present in approximately the same percentage (25%) of trees from the posterior distribution (Fig. 3). Two pairings (*A. pygmaea* most closely related to *A. cristatella* and *A. psittacula* most closely related to *A. pusilla*) appeared in less than 5% of the trees, and 55% of trees showed *A. cristatella* as sister to *A. pusilla*.

Using the data matrix that combined our ND2 data with Pereira and Baker's (2008) data, we recovered two different topologies. Under the GTR + I + Γ model, Node A had a posterior probability of 0.86, while Node B was not recovered (thus, the topology showed a three-species polytomy); these values increased to 1.0 (Node A) and 0.97 (Node B) under the GTR + SS model. When we included only 3 of our ND2 sequences per species (to control for the possibility that our ND2 dataset was driving the phylogenetic inference), Node A had a posterior probability of 0.99 (under the GTR + I + Γ model), and Node B had a posterior probability of 0.63 (under the same model). When the truncated data matrix was analyzed using the GTR + SS model, the posterior probabilities were 1.0 (Node A) and 0.98 (Node B). This pattern was retained when only one ND2 sequence was used. This arrangement of auklet taxa is the same of one of the two possible arrangements Pereira and Baker (2008) found using their concatenated data set under a GTR + I + Γ model (they did not use a GTR + SS model); neither arrangement was well-supported in their analysis. (We also analyzed the concatenated dataset using a partitioned model, with a different model chosen for each gene. The results were not different from a non-partitioned model and are not presented separately.)

The RAG-1 dataset recovered two other topologies (trees not shown); under the GTR + I + Γ model, *A. psittacula*, *A. cristatella*, and *A. pygmaea* formed a three-species polytomy with a posterior probability of 0.68. However, under the GTR + SS model, *A. psittacula* and *A. cristatella* were recovered as sister, with a posterior probability of 0.82. This clade was sister to *A. pygmaea*; the posterior probability of this node was 0.78. (Pereira and Baker (2008) concatenated their mtDNA and RAG-1 sequences and did not examine the RAG-1 dataset by itself.)

The ND2 BEAST tree recovered *A. psittacula* and *A. pygmaea* as sister species (4). *A. cristatella* was sister to this clade, and *A. pusilla* was basal to the rest of *Aethia*.

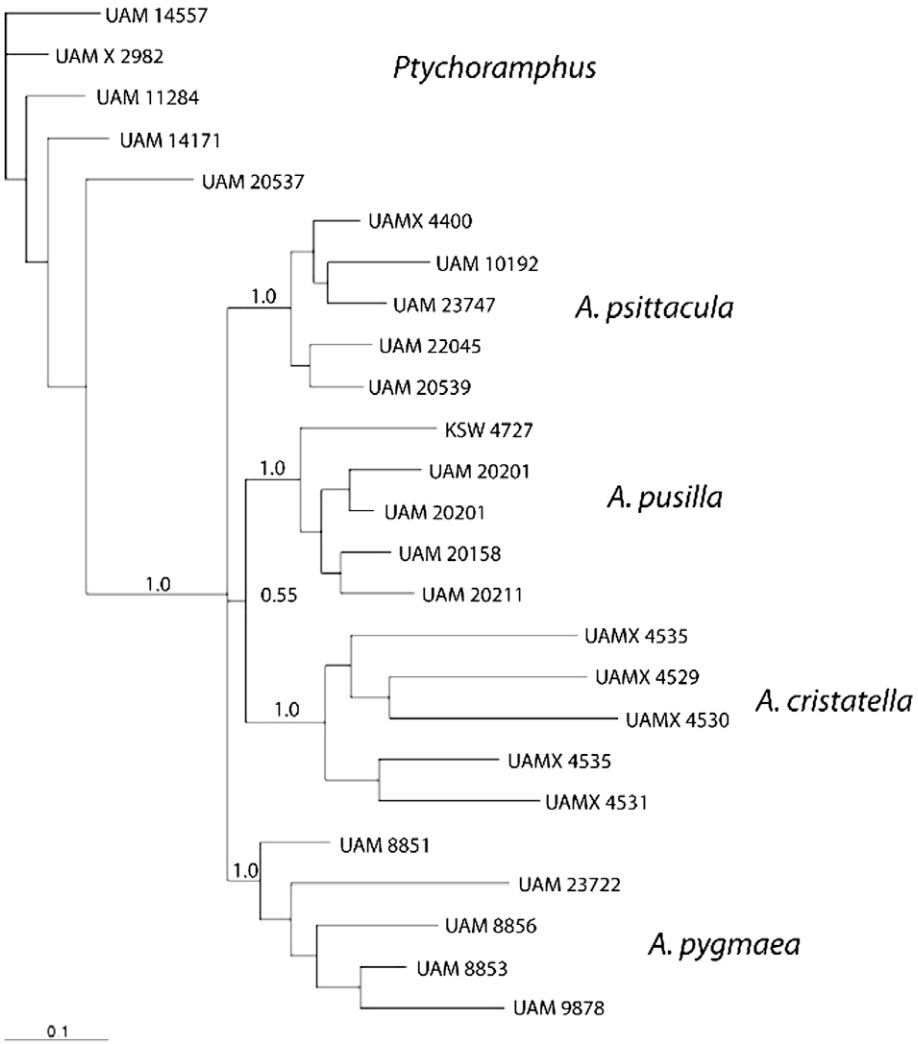

Fig. 2. Bayesian AFLP phylogeny. Phylogeny reconstructed from 898 AFLP loci using the restriction site (binary) model in MrBayes ver. 3.1.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003).

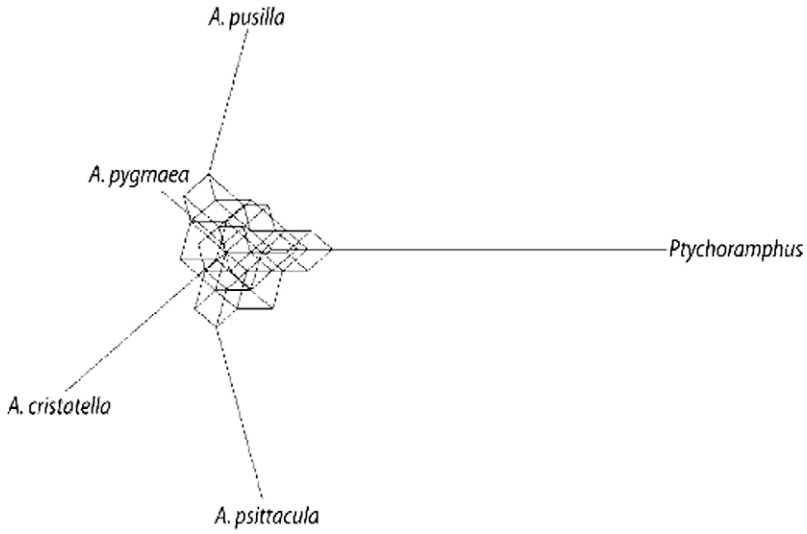

Fig. 3. Network of possible sister relationships (AFLP data). Relationships that appear in more than 5% of trees in the posterior distribution are included. Each vertex represents another possible data bipartition. Only relationships between the species are shown.

3.2. Contribution of increased taxon sampling

Under the better molecular model (i.e., the model that had a lower log-likelihood score; GTR + SS) on our ND2 dataset, the posterior probability of Node A was unaffected by sample size (Fig. 5). For Node B (the more weakly supported node), trees with a sample size of 3 or more showed more robust support than trees with a sample size of only 1 or 2. Additionally, when we sampled only 1 or 2 individuals per species, 1 replicate of the 5 failed to return a topology congruent to the topology returned by the full ND2 dataset (not shown).

3.3. Estimation of the length of the Aethia radiation

The most recent common ancestor (MRCA) of the auklets (genera *Ptychoramphus* and *Aethia*) was estimated to have occurred 64.79 standard time units (STU) ago, with a 95% highest posterior density (HPD) of 52.61–77.69 STU (Fig. 4). (An HPD is a set of intervals that enclose 95% of the posterior distribution.) The genus *Aethia* shared a MRCA 51.10 (40.18–61.48) STU ago. Within *Aethia*, the TMRCA for Node A was 45.52 (35.44–56.25) STU. Node B on the MrBayes ND2 tree (Fig. 1) did not exist in the BEAST tree; instead, BEAST placed *A. cristatella* and *A. psittacula* as sister species, with *A.*

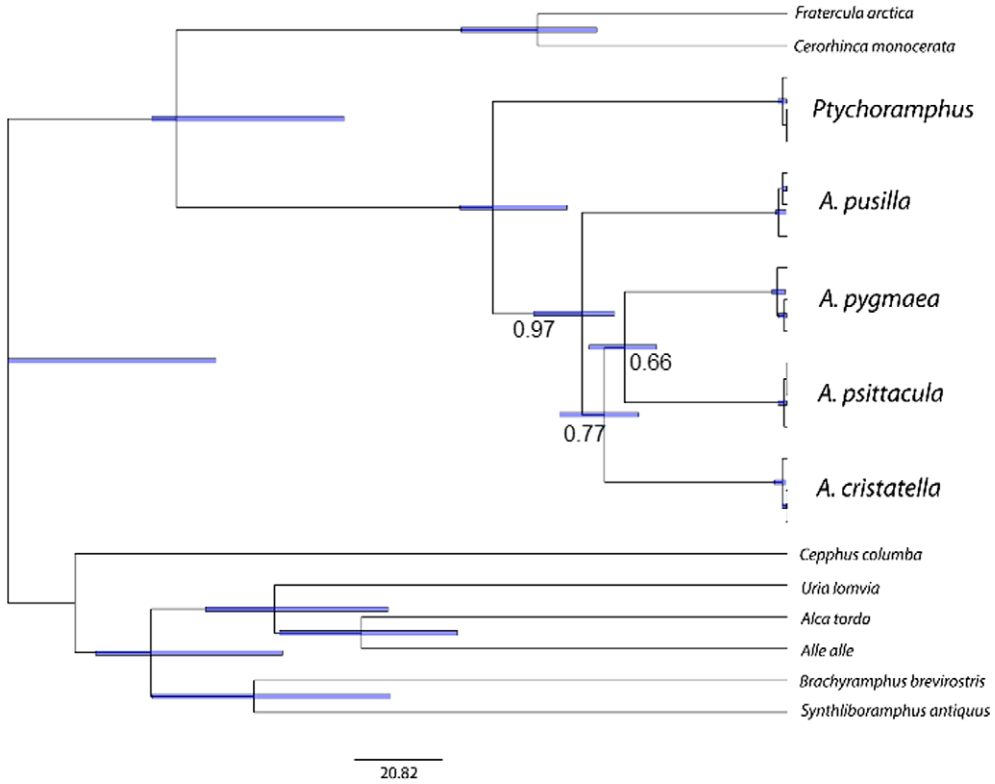

Fig. 4. Tree (ND2 data). The gray boxes show the 95% highest posterior density of estimated times to most recent common ancestors. Branches are scaled to estimated number of substitutions per 1041 base pairs of mtDNA sequence (the length of the ND2 gene). The numbers underneath the branches leading to the *Aethia* species are posterior probabilities.

pygmaea as basal. The TMRCA for *A. cristatella* and *A. psittacula* was 38.87 (31.40–47.60) STU. The *Aethia* radiation lasted for a total of 12.23 (8.79–13.88) STU.

4. Discussion

4.1. Aethia phylogeny

We were able to infer a fully bifurcating, resolved phylogeny using our ND2 dataset (Fig. 1). Under some models, the GenBank data matrices corroborated this topology, although the full matrix analyzed using a GTR + I + Γ model returned an unresolved topology. Morphological evidence matches partially with the topology recovered from our ND2 dataset. *A. cristatella* and *A. pygmaea* were recovered as sisters in our mtDNA tree. Morphologically, these two species were assumed to be sister because they share several traits (facial plumes, a citrus odor, and vocalizations) that are not found in any of the other true auklet species (Jones, 1993a). However, morphology also suggested that *A. pusilla* is more closely related to the presumed *A. cristatella–A. pygmaea* clade (Jones, 1993b). Our mitochondrial data suggest instead that *A. psittacula* is closer to the *A. cristatella–A. pygmaea* clade. If the true species relationship matches the one inferred by the mitochondrial genome, then the morphological evidence suggesting a closer relationship between *A. pusilla* and *A. pygmaea* (size, mostly, but also bill color) is the result of one of three events: a loss of phenotypic characters on the *A. psittacula* branch; a loss of phenotypic characters in the ancestor of the *A. psittacula–A. cristatella–A. pygmaea* clade, followed by a reversal in the ancestor for the *A. cristatella–A. pygmaea* clade; or convergence between *A. pusilla* and the *A. cristatella–A. pygmaea* clade.

A phylogeny built using just mtDNA is not necessarily representative of the true species phylogeny (Ballard and Whitlock, 2004). The relationships supported by a single locus may be the actual species relationships, but they can also be the result of stochastic lineage sorting or a selective sweep (Ballard and Whitlock, 2004). Additionally, mtDNA is maternally inherited and thus can cross species boundaries through interspecific hybridization. If widespread hybridization occurred soon after diversification, the "wrong species" mtDNA could have easily become fixed; in this case, the evolutionary history of mtDNA would not be the evolutionary history of the species (Ballard and Whitlock, 2004). Ideally, we could test whether the relationships inferred by mtDNA are correct using an independent dataset, such as from nuclear DNA. However, both phylogenies inferred using two nuclear data sets, the RAG-1 dataset from Pereira and Baker (2008) and our AFLP dataset, had two completely different topologies, neither of which matched the mtDNA topology. In the case of Pereira and Baker's (2008) RAG-1 data, *A. psittacula* and *A. cristatella* were found to be sister taxa; analysis of our AFLP data set returned an unresolved tree (Fig. 2). The lack of concordance among the various gene trees is not necessarily unexpected. The mitochondrial and nuclear genomes are inherited independently and have different rates of sorting (Moore, 1995). Lineage sorting can cause different loci to infer different relationships, especially when the lengths of internal branches are short, as we saw in all resolved *Aethia* phylogenies. This is the explanation favored by Pereira and Baker (2008). Gene trees are not species trees, and the probability of a 5-taxa gene tree being discordant with the species tree is high, especially when the internode lengths are short. It is possible that none of the three topologies supported by the three genetic datasets is the species tree.

Why would we get a resolved tree with mtDNA and nuclear sequence data (under one model) but still see a polytomy with an AFLP sampling of the whole genome? One possible explanation is that we have not generated enough AFLP loci for phylogenetic analysis. However, 70% of our 898 loci were polymorphic. In other studies (*Pinus pinaster*, Ribiero et al., 2002; *Solanum* L. section *Lycopersicon*, Spooner et al., 2005), ~1000 or fewer loci with a smaller percentage of polymorphisms have provided enough signal to resolve shallow phylogenetic relationships. AFLPs may also a poor marker for birds; we do not think this is the case because AFLPs have been used very effectively in population-level studies of house sparrows (Wang et al., 2003) and crossbills (Parchman et al., 2006). Another possible explanation for the polytomous topology recovered in our Bayesian consensus tree of the AFLP data is the stochastic process of lineage sorting. Based on our mtDNA coalescent analyses, the speciation events occurred so closely together that our estimated 95% HPDs overlapped (Fig. 4). The stochastic process of DNA lineage sorting during short internode time intervals is notorious for causing different genes to have different evolutionary histories (Avise, 2004). With a method such as AFLPs, which amplifies stretches of DNA over many different genes, conflicting phylogenetic signals across the genome could result in a consensus topology that either showed a polytomy or very low nodal support.

Another possible explanation is that the *Aethia* auklets are too divergent for the AFLP technique to be useful in resolving the phylogeny. As species become more divergent, lack of homology of generated fragments becomes a serious issue (see Lerceteau and Szmidt, 1999; Robinson and Harris, 1999). Bremer (1991) suggested that treating non-homologous loci as homologous artificially increases homoplasy and thus obscures any phylogenetic signal in RFLP data. AFLPs may be subject to similar problems. Lerceteau and Szmidt (1999) discovered that AFLPs failed when attempting to reconstruct the deeper relationships within the genus *Pinus*, although the method was successful in recovering shallower relationships. Our AFLP data showed that the Bayesian consensus tree (without collapsed branches) recovered a relationship not found in any mtDNA or morphological tree (*A. cristatella* and *A. pusilla* as sister taxa). With the exception of the bipartitions containing *A. cristatella*, all other possible pairwise relationships were supported in approximately 25% of the trees of the posterior distribution; this same result would occur if we randomly assigned AFLP locus scores to each species (assuming we only sampled one individual per species). However, two lines of evidence suggest that the AFLP technique is not at fault: (1) all individuals were correctly assigned to their respective species, and each species formed a monophyletic clade; and (2) the lengths of the branches leading to each species clade were not the same. We suspect that the branches would be the same length if locus states had truly been randomly assigned.

4.2. Contribution of increased sample size toward resolution of the polytomy

Fully 20% ($N = 5$) of phylogenetic reconstructions using ND2 and one or two individuals recovered a tree that was incongruous with the tree recovered using the full dataset (Fig. 5, Node B). Using ND2, it appears that for divergences at this depth adding individuals past three per taxon only increased the intraspecific variation. Maddison and Knowles (2006) found this same relationship with a simulated dataset. No matter how many loci they used to build their trees, the accuracy of species tree inference plateaued when three or more individuals were sampled. Felsenstein (2006) also noticed this relationship when calculating phylogeny-based likelihood parameters. Our results provide additional, empirical evidence.

4.3. Estimation of times to most recent common ancestor (TMRCA) between species

The range of the *Aethia* radiation can be calculated by subtracting the estimate of TMRCA for the most recent split (in this case,

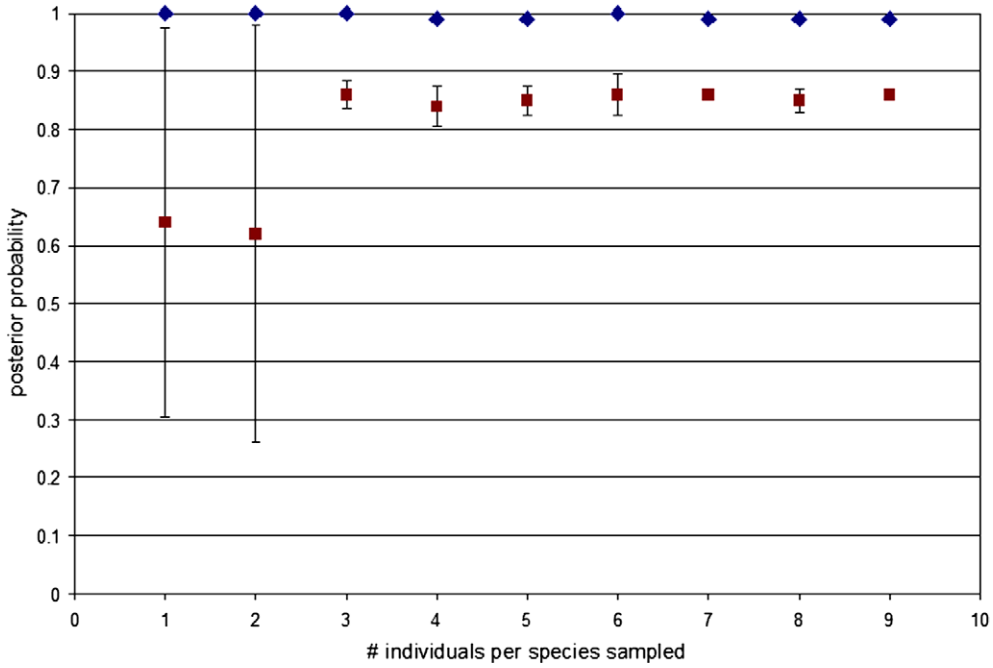

Fig. 5. Change in posterior probabilities of nodes. Posterior probabilities were calculated under the GTR + SS model. Node A (shown in blue) is the node mat unites *A. pygmaea, A, cristatella,* and *A.* psittacula; and Node B (shown in red) unites *A. pygmaea* and *A. cristatella* (Fig. 1). We sampled a minimum of 1 individual per species and a maximum of 9 individuals per species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the *A. pygmaea–A. psittacula* divergence) from the estimate of TMRCA for the entire genus. We can convert STU into years if we multiply the estimates by a substitution rate. Previous estimates suggested that the auklet radiation occurred over a very short period (less than 100,000 years; Walsh et al., 1999), but our estimates suggest that it took 800,000 years at the very least (assuming the overall mtDNA genome clock of 2.7% per million years from Walsh et al., 1999). If we use a standard mtDNA clock of 2.1% per million years (Weir and Schluter, 2008), the radiation would have taken place over 1.18 million years, and using the clock of 2.61% per million years (estimated for Charadriiformes; Weir and Schluter, 2008) changes the radiation range to 900,000 years long. Doubling the fastest mutation rate (see Ho et al., 2007) only decreases the estimated range by half, to 435,000 years long. (This doubled rate is faster than the fastest rate Weir and Schluter (2008) found among the Charadriiformes datasets examined.) In fact, we would have to use an unlikely mutation rate of 122.3% to confine our coalescence-estimated range of this radiation to 100,000 years. Thus, there is no realistic molecular clock that can limit the estimated length of the *Aethia* radiation to the 100,000 years suggested by power analysis.

However, the reality of the molecular clock has been questioned, and in birds the standard clock has been calibrated in some lineages, including Alcidae (Weir and Schluter, 2008). Pereira and Baker (2008) used fossil calibration points to estimate the ages of the most recent common ancestor of the *Aethia* auklets, a method that does not require a clock estimate. Their estimated length of the radiation (calculated using the means of their posterior distributions) was still around 1 million years, a 10-fold increase from the Walsh et al. (1999) power-analysis estimate of 100,000 years.

A tree made using TMRCA estimates produced a topology different from that obtained using MrBayes. However, because our HPD intervals overlapped, any topology created using only TMRCA estimates is suspect. Although hard or near-hard polytomies have been shown to mislead Bayesian inference (Lewis et al., 2005; Steel and Matsen, 2007), the topology we recovered using Bayesian methods was also recovered using both parsimony and maximum likelihood (not shown). We feel confident that we are not seeing a Bayesian "star paradox" (Steel and Matsen, 2007) with our ND2 data.

5. Conclusion

Polytomies can exist both at the gene/locus level and at the species level. In the *Aethia* auklets, the mtDNA phylogeny of Walsh et al. (1999) showed a gene-level polytomy, although the true *Aethia* species tree may be polytomous. We were able to confidently resolve the mtDNA polytomy when using a different gene from the same locus (ND2), a larger concatenated dataset, better analytical techniques using improved models of molecular evolution, and by sampling more than a single individual per species. We were unsuccessful in reconstructing a bifurcating species-level phylogeny of the *Aethia* auklets using AFLPs. The technique may not be useful for species that are 7–9% divergent in mtDNA or that have short internode distances at that depth. The polytomous AFLP consensus tree may also reflect a history of incomplete lineage sorting among the *Aethia* auklet species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.07.023.

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