Molecular phylogenetics of the wrens and allies (Passeriformes: Certhioidea), with comments on the relationships of Ferminia

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ABSTRACT

The superfamily Certhioidea is distributed on four continents and while comprising relatively few species, includes forms as diverse as creepers, nuthatches, gnatcatchers, and wrens. Previous attempts to infer the phylogeny of this lineage have focused on its higher-level relationships, consequently undersampling the New World wrens. This study reports the first nearly genus-level sampling of certhioids, based on concatenated and species tree analyses of 8520 bases of DNA sequence data from six gene regions. These analyses, while failing to completely resolve basal certhioid relationships, corroborate the monophyly of a diverse New World clade of gnatcatchers, gnatwrens, and wrens, and significantly improve our understanding of wren relationships. The inferred relationships among certhioids and wrens support an Old World origin for these lineages, with dispersal of the New World clade in the mid-Miocene, suggesting expansion and early diversification of the lineage through North America. This scenario suggests a minimum of six independent dispersal events into South America in this lineage, at least some likely to have been made prior to the Pliocene.

INTRODUCTION

PREVIOUS HYPOTHESES OF RELATIONSHIP WITHIN CERTHIOIDEA

The superfamily Certhioidea is a small clade of passerine birds comprising the families Sittidae (nuthatches and the wallcreeper), Certhiidae (creepers), Polioptilidae (gnatcatchers and

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gnatwrens), and Troglodytidae (wrens). The monophyly of this group of approximately 141 species (Gill and Donsker, 2017) was first indicated by analyses of DNA-DNA hybridization data (Sibley and Ahlquist, 1990; fig. 1). These analyses also suggested the group was most closely related to Old World warblers, bulbuls, titmice, and relatives, leading to its placement within the oscine passerine superfamily Sylvioidea. Subsequent analyses of family-level passerine nuclear sequence data questioned placement in Sylvioidea, instead suggesting recognition at the superfamily level (Barker et al., 2002; Barker et al., 2004; Cracraft et al., 2004). More recent molecular work has consistently supported monophyly of members of this clade (Alstrom et al., 2006; Johansson et al., 2008; Fregin et al., 2012; Moyle et al., 2016; Zhao et al., 2016), although to date few studies have included representatives of all its major lineages.

As for most passerine families, this superfamily has no known morphological synapomorphies. That the close relationship of species in the group was not previously recognized is likely due to the fact that it comprises at least four major ecotypes with differing locomotory and feeding specializations that do not appear entirely concordant with phylogeny. In the hind limb, the group is split between strong graspers that can cling to branches, vertical trunks, or even sheer stone surfaces (nuthatches, the wallcreeper, and some wrens) and relatively weak graspers that cling to trunks with tail support (creepers) or are limited to branches or horizontal surfaces (gnatcatchers, gnatwrens, and some wrens). In the rostrum, the group is split among species with long narrow mandibles specialized for reaching prey in crevices (creepers, the wallcreeper, gnatwrens, and most wrens), those with short broad bills that are used to excavate prey and even nest cavities (nuthatches), and those with delicate surface gleaning bills (gnatcatchers and some wrens). This morphological and ecological diversity led early taxonomists to associate these groups with many convergently similar lineages, including the Australasian sittellas (Daphoenositta) and treecreepers (Climacteris), the Philippine creeper (Rhabdornis), the Malagasy coral-billed nuthatch (Hypositta), and the Old World warblers (Sylviidae sensu lato, now Sylviidae, Acrorhynchidae, Phylloscopidae, etc.; Alstrom et al., 2006; Fregin et al., 2012).

By contrast, relatively little consensus has been reached regarding relationships within the group. Perhaps the strongest single result has been monophyly of the New World lineage of Certhioidea, comprising the Poliopiilidae and Troglodytidae. Every study including both lineages (e.g., Sibley and Ahlquist 1990; Barker et al., 2004; Barker 2004; Alstrom et al., 2006; Johansson et al., 2008; Zhao et al., 2016) has found strong support for their sister-group relationship, although Sibley and Ahlquist (1990) found the New World verdin (Auriparus) sister to the Poliopiilidae, apparently due to a lab error or sample misidentification (subsequent studies have found Auriparus related to the penduline tits; e.g., Sheldon and Gill, 1996; Johansson et al., 2008). The most consistently sampled lineages other than the wrens and gnatcatcher clade (the New World Certhioidea, NWC) have been creepers (Certhia) and nuthatches (Sitta). All three possible relationships among these two genera and the New World lineage have been recovered in various studies: NWC+Certhia (Barker et al., 2002; Moyle et al., 2016; Zhao et al., 2016), NWC+Sitta (Alstrom et al., 2006; Johansson et al., 2008), and Sitta+Certhia (Barker et al., 2004). In terms of number of data sets (the three noted above) and data set size (4155 loci in Moyle et al., 2016), the evidence appears to be in favor of a NWC+Certhia relationship.
Placement of the enigmatic genera *Salpornis* (the spotted creeper) and *Tichodroma* (the wall-creeper) relative to the NWC and *Certhia* remains somewhat in question: only three studies to date have included the former and only two the latter. The three studies including *Salpornis* have placed it either as sister to *Sitta* (Johansson et al., 2008) with weak support (although with strong support separating *Salpornis* from *Certhia*), or as sister to *Certhia* (Sibley and Ahlquist, 1990; Zhao et al., 2016) with strong support (or unevaleuable support, in the case of DNA-DNA hybridization tree). The two studies to date that have placed *Tichodroma* phylogenetically (Sibley and Ahlquist, 1990; Zhao et al., 2016) have supported its relationship with *Sitta* (with strong support in the latter study), as expected by previous morphological and behavioral evaluations (reviewed in Vaurie, 1957; Sibley and Ahlquist, 1990). Excepting the placement of *Auriparus*, the current consensus on certhioid relationships looks essentially the same as that of Sibley and Ahlquist in 1990 (fig. 1).

**Hypotheses of Relationship within Troglodytidae**

The wrens are the most diverse lineage of certhioids, comprising at least 84 species (60% of the superfamily) in 19 genera (Gill and Donsker, 2017). Despite this diversity, to date only a few studies have addressed higher-level relationships within the group in any detail. Sibley and Ahlquist sampled only eight species in as many genera (fig. 1), and found relatively little structure among them, with *Microcerculus* falling out as most divergent, and a close relationship between the Carolina (*Thryothorus ludovicianus*) and Bewick’s (*Thryomanes bewickii*) wrens. Overall divergence within wrens had a maximum of $\Delta T_{50} = 6.0$, roughly suggesting a clade age of ~14 Ma (assuming 2.35 Ma/$\Delta T_{50}$, half the value for nonpasserines; Sibley and Ahlquist, 1990). More recent work on wren relationships based on DNA sequence data has improved our understanding of wren relationships (summarized in fig. 2). Barker (2004) showed: (1) the root of the wren tree most likely lay among a grade of highly terrestrial wrens including *Salpinctes, Catherpes, Hylorchilus*, and *Microcerculus*; (2) a close relationship between the genera *Cistothorus* and *Troglodytes*; (3) a close relationship between *Campylorhynchus* and *Thryomanes/Thryothorus ludovicianus*; (4) paraphyly of *Thryothorus* as recognized at the time; and (5) a well-supported relationship of “Thryothorus” except the type (*T. ludovicianus*) with the wren genera *Cyphorhinus, Henicorhina, Uropsila*, and *Cinnycerthia*. Subsequent work by Mann et al. (2006) extending sampling of “Thryothorus” to nearly all species of the group corroborated previous results and showed that all members of the genus except the type fell into three major clades more closely related to other genera than to the type. Two of those clades had available generic names that were resurrected, and Mann et al. erected a new genus for the third. Additional work on the genus *Troglodytes* and allies (Rice et al., 1999; Gómez et al., 2005) has shown that: (1) the Timberline Wren (*Thryorchilus browni*) is a close relative of the genus, (2) the Winter Wren may best be recognized in its own genus, *Nannus* (although evidence for its exclusion from *Troglodytes* is not overwhelming); and (3) the Socorro Wren, traditionally placed in *Thryomanes*, is actually a member of *Troglodytes*. 
FIGURE 1. Phylogeny of the Certhioidea based on DNA-DNA hybridization (Sibley and Ahlquist 1990), scaled by a measure of genetic divergence ($\Delta T_{50}^H$). The quotes around genus *Auriparus* indicate that its placement is artifactual (possibly a labelling error; see text). Closed circles indicate estimated Bayesian posterior probabilities $\geq 0.95$ for equivalent nodes (taxon sampling differs) in the concatenated gene analyses of Zhao et al. (2016).

**Focus of This Study**

Although molecular studies have advanced our understanding of certhioid relationships, only two studies have sampled all the relevant deep lineages, and none has densely sampled the most diverse lineage, the wrens. This study addresses both of these gaps. First, I infer a hypothesis of higher-level certhioid relationships including all major lineages, based on a six-gene (one mitochondrial, five nuclear) data set. Second, based on the same data, I infer the first genus-level phylogeny of the wrens (family Troglodytidae). Finally, I review some implications of this phylogeny for the evolution of wrens.

**MATERIALS AND METHODS**

**Taxon and Gene Sampling:** This study samples all but one of the currently recognized genera of Certhioidea (Gill and Donsker, 2017), lacking only the polioptilid *Ramphocaenus*, which is closely
related to *Microbates* (Barker, unpublished data). This includes samples from all four clades of the previously recognized genus "*Thryothorus*" (true *Thryothorus*, *Pheugopedius*, *Thryophilus*, and *Cantorchilus*; Mann et al., 2006), both *Troglodytes* sensu stricto and *Nannus* (the Winter Wren, which some consider generically distinct; Rice et al., 1999; Gómez et al., 2005), and the monotypic Caribbean endemic genus *Ferminia* (table 1), which has never before been included in a phylogenetic study. Outgroups for this analysis included members of the Donacobiidae, Cisticolidae, Zosteropidae, Mimidae, Sturnidae, and Turdidae (table 1), as in a previous study of wren relationships (Barker, 2004). In terms of loci, this study includes sequences from one mitochondrial (cytochrome *b*) and five autosomal nuclear gene regions: RAG1 and RAG2 (recombination activating genes 1 and 2, respectively), FGB (β-fibrinogen, introns 4 and 7), and ZEB1 (zinc finger E-box-binding homeobox 1). All of these loci except ZEB1 (HomoloGene UID #31779, formerly known as TCF8 and δEF1) have been used previously in avian systematics (e.g., Prychitko and Moore, 1997; Barker, 2004; Barker et al., 2004). Similar to RAG1, ZEB1 is a strongly conserved gene with a long exon (though not the sole exon, as in RAG1) that preliminary results indicate is useful in avian phylogenetics (Herreman, 2000).
| Family              | Taxon                          | Sample | CYTB    | FGB-I4  | FGB-I7  | RAG1    | RAG2    | ZEB1    |
|---------------------|--------------------------------|--------|---------|---------|---------|---------|---------|---------|
| Donacobiidae        | Donacobius atricapilla         | FMNH1772 [SML88-246, MPEG] | AY352528 | AY352559 | MG495516 | AY319979 | MG495488 | MG495435 |
| Cisticolidae        | Prinia bairdii                 | FMNH355824 | AY352536 | AY352567 | MG495527 | AY319998 | AY799845 | MG495446 |
| Zosteropidae        | Zosterops senegalensis         | FMNH346671 | AY352549 | AY352580 | MG495540 | AY057042 | AY443247 | MG495459 |
| Troglodytidae       | Campylorhynchus brunnecapillus | FMNH342076 | AY352520 | AY352551 | MG495509 | MG495460 | MG495482 | MG495428 |
|                     | Odontorchilus cinereus         | FMNH1789 [DW3682, MPEG] | AY352533 | AY352564 | MG495524 | MG495472 | MG495496 | MG495443 |
|                     | Salpinctes obsoletus           | UWBM103855 |          |         | MG495409 | MG495422 | MG495528 | MG495474 |
|                     | Catherpes mexicanus            | FMNH5470 [BEHB033, MZAH] | AY352522 | AY352553 | MG495511 | MG495462 | MG495484 | MG495430 |
|                     | Hylorchilus sumichrasti        | MZAH9734 | AY352531 | AY352562 | MG495520 | MG495468 | MG495492 | MG495439 |
|                     | Cimnyerthia peruana            | LSUMZB-1860 |          |         | MG495401 | MG495415 | MG495513 | MG495463 |
|                     | Cistothorus platensis          | MMNH47271 |          |         | MG495402 | MG495416 | MG495514 | MG495464 |
|                     | Thryomanes bewickii            | MZAH9734 | AY352541 | AY352572 | MG495533 | MG495476 | MG495502 | MG495452 |
|                     | Ferminia cerverai              | AMNH300419 |          |         | MG495404 | MG495418 | MG495518 | MG495466 |
|                     | Pheugopedius genibarbis        | FMNH427189 |          |         | MG495408 | MG495421 | MG495525 | MG495473 |
|                     | Thryophilus rufalus            | FMNH434072 |          |         | MG495411 | MG495424 | MG495534 | MG495477 |
|                     | Cantorhichus longirostris      | FMNH392954 |          |         | MG495400 | MG495414 | MG495510 | MG495461 |
|                     | Thryothorus ludovicianus        | KUMNHB-2137 [MZAH] | AY352545 | AY352576 | MG495536 | MG495479 | MG495505 | MG495455 |
|                     | Nannus troglodytes              | UWBM300419 [CWS6240] |          |         | MG495407 | MG495420 | MG495523 | MG495471 |
|                     | Troglodytes aedon              | FMNH343273 | AY352547 | AY352578 | MG495538 | AY057038 | AY443241 | MG495457 |
|                     | Thryorchilus browni            | LSUMZB-19933 | AY352542 | AY352576 | MG495535 | MG495478 | MG495504 | MG495454 |
|                     | Uropsila leucogastra           | Unvouched | DQ415712 | NA       | NA       | NA       | NA       | NA       |
### Generation of Molecular Data:

Genomic DNA was extracted from all samples using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). DNA from the sample of *Ferminia* was subsequently amplified by random priming using the illustra GenomiPhi V2 amplification kit (GE Healthcare, Pittsburgh, PA) to obtain adequate amounts of high molecular weight DNA. All loci were amplified by polymerase chain reaction using previously described primers and cycling conditions (Barker, 2004; Barker et al., 2004; Kimball et al., 2009), although some PCRs for this study were performed using GoTaq G2 Hot Start Master Mix (Promega, Madison, WI). To obtain complete sequences for *Ferminia*, additional specific primers were created for all loci (appendix 1). For most taxa, ZEB1 was amplified in three fragments using the primer pairs EF31F/EF799R, EF678F/EF1356R, and dEF1x/EF1707R (or in some cases EF_3prime; fig. 3, table 2), using a touchdown cycling profile (five cycles each at 58° C, 56° C, 54° C annealing temperature, followed by 20 at 52° C annealing), and 1 minute extension times. PCR reactions

| Family         | Taxon                        | Samplea | CYTB       | FGB-I4   | FGB-I7   | RAG1   | RAG2   | ZEB1     |
|----------------|------------------------------|---------|------------|----------|----------|--------|--------|----------|
| Polioptilidae  | Microbates collaris         | AMNH    | MG495406   | MG495419 | MG495521 | MG495469 | MG495493 | MG495440 |
|                | Polioptila caerulea         | FMNH    | MG495410   | MG495423 | MG495529 | MG495475 | MG495499 | MG495448 |
| Sittidae       | Sitta pygmaea               | FMNH    | MG495410   | MG495423 | MG495537 | MG495530 | MG495506 | MG495456 |
| Tichodromidae  | Tichodroma muraria          | UWBM    | MG495413   | MG495426 | MG495530 | MG495500 | MG495449 |
| Certhiidae     | Certhia americana           | FMNH    | MG495410   | MG495423 | MG495529 | MG495475 | MG495499 | MG495448 |
| Mimidae        | Dumetella carolinensis      | FMNH    | MG495420   | MG495423 | MG495529 | MG495475 | MG495499 | MG495448 |
| Sturnidae      | Sturnus vulgaris            | FMNH    | MG495420   | MG495423 | MG495529 | MG495475 | MG495499 | MG495448 |
| Turdidae       | Sialia sialis               | FMNH    | MG495410   | MG495423 | MG495529 | MG495475 | MG495499 | MG495448 |

* Institutional abbreviations: AMNH (American Museum of Natural History), FMNH (Field Museum of Natural History), KU (Biodiversity Institute, University of Kansas), LSUMZ (Museum of Zoology, Louisiana State University), MZAH (Museo de Zoología “Alfonso L. Herrera,” Universidad Nacional Autónoma de México), UWBM (Burke Museum, University of Washington).
were evaluated by agarose gel electrophoresis, and successful amplifications with appropriately sized products were submitted to Beckman-Coulter Genomics (Danvers, MA) for clean up and sequencing with BigDye Terminator v3.1 on an ABI 3730 automated sequencer, using external and internal primers, as necessary. Individual reads were assembled and edited in Geneious v5.6.7, then exported to text for alignment and subsequent analysis.

**Alignment and Phylogenetic Analyses:** Sequences for each gene region were aligned using MUSCLE v3.6 (Edgar, 2004) with default parameters, then concatenated for analysis. Certhioi phylogenies were estimated using single gene, concatenation, and species tree analyses in both likelihood and Bayesian frameworks. Likelihood searches as implemented in RAxML v7.0.3 (Stamatakis, 2006) were used to estimate a concatenated gene phylogeny, with a partitioned GTR+G model allowing proportional branch lengths among partitions. The partitioning scheme was selected using Partitionfinder v1.1.1 (Lanfear et al., 2012), using the greedy search algorithm and BIC as an optimality criterion, and starting with 14 partitions: all codons of each protein coding gene (cytochrome b, RAG1, RAG2, and ZEB1) separately, plus both introns (FGB-I4, FGB-I7). A search for the best tree was made 10 times from random starting points, and nodal support was assessed by 1000 bootstrap replicates (Felsenstein, 1985) using the fast search option. The same partitioning scheme (and model parameterization) was used to estimate relationships for the concatenated data set using Bayesian methods as implemented in MrBayes v3.2.5, using default priors for all parameters except for branch lengths, which were set to an exponential prior with a rate of 100, in order to avoid long branch artifacts identified in initial runs (Marshall, 2010). Bayesian methods were also used to generate gene-specific estimates of phylogeny for comparison with the combined estimate, and to assess among-gene heterogeneity in phylogenetic estimates. For each Bayesian analysis, I performed two MCMC runs each of 2×10^6 generations, sampling every 100. Adequate (≥200) effective sample size for parameters, parameter convergence, and burn-in were determined using Tracer v1.5, and overall topological and nodal convergence was assessed using functions of the “rwty” package (Lanfear et al., 2016) in R (R Core Team, 2016). A maximum clade credibility tree was calculated from the combined output using TreeAnnotator v1.8.3.

Taxonomic hypotheses of interest were evaluated on the concatenated data by comparing the marginal log-likelihood estimates from unconstrained analyses to values from constrained analyses enforcing monophyly of specific clades using empirical Bayes factors (Kass and Raftery, 1995). Hypotheses evaluated included: (1) monophyly of “Thryothorus” (Thryothorus, Pheugopedius, Thryophilus, and Cantorchilus); (2) monophyly of all former Thryothorus except T. ludovicianus; (3) broad-sense monophyly of Troglydotes (including Nannus, Troglydotes, and Thryorchilus); and (4) monophyly of Troglydotes in the strict sense (Nannus and Troglydotes only). Marginal log-likelihoods were estimated via stepping-stone analyses (Xie et al., 2011) in
TABLE 2. Novel primers used in amplification of ZEB1.

| Primer Name   | Direction | Sequence                        | Source                                      |
|---------------|-----------|---------------------------------|---------------------------------------------|
| dEF1x         | forward   | CTTCTGGACCATCTTCTCCTGA          | G.F. Barrowclough and J.G. Groth, personal commun. |
| EF31F_new     | forward   | GCCATATGAGYGCCCCAATGCAA         | modified from Barrowclough and Groth, personal commun. |
| EF678F        | forward   | GTCAACTTCAGGTGTTCACA            | G.F. Barrowclough and J.G. Groth, personal commun. |
| EF799R_new    | reverse   | GACARTCATCAAAAGAGACAA           | modified from Barrowclough and Groth         |
| EF_3prime     | reverse   | CTTGGGTCTGTTTTGGTGGT           | G.F. Barrowclough and J.G. Groth, personal commun. |
| EF1356R       | reverse   | GTGGCTCTTCTTGTACACCCTC         | G.F. Barrowclough and J.G. Groth, personal commun. |
| EF1707R       | reverse   | GCCAATAAGCAAACCATTTTGATTCACC   | Herreman 2000                                |

MrBayes, using 50 steps and $\alpha = 0.4$, sampling $1 \times 10^8$ generations, with burn-in set to equal the number samples in each step (burninss=-1).

In addition to single gene and concatenated analyses, I estimated certhioid phylogeny using Bayesian species-tree methods as implemented in *BEAST v1.8.3 (Heled and Drummond, 2010). Species tree analyses assume free recombination between loci, but no recombination within loci, essentially treating each gene rather than each site as an independent measure of species relationships (Liu et al., 2008; Heled and Drummond, 2010). Since two pairs of the six loci included in this study—FGB-I4/FGB-I7 and RAG1/RAG2—are closely physically linked (separated by 1251 and 8042 bases respectively in *Taeniopygia; GenBank assembly 3.2.4, Annotation release 101), I treated these pairs as single loci in terms of topology (but not in substitution model), yielding effectively four independent loci for the purposes of species tree analysis. Ploidy was set as mitochondrial for cytochrome b, and autosomal for the remaining loci, based on assumed synteny with *Taeniopygia. I set a Yule prior on the species tree topology, used a piecewise constant multispecies coalescent model, and assumed an uncorrelated log-normally distributed model of lineage-specific rate variation. Priors on gene-specific rates were set as exponential distributions with means of 0.1. I performed two MCMC runs each of $1 \times 10^8$ generations, sampling every 5000. Run outputs were analyzed as for the concatenated and single gene analyses reported above.

It was previously shown that RAG1 and RAG2 sequences of *Sitta deviate strongly from stationarity, showing an excess of GC nucleotides at third-codon positions in comparison with other passerines (Barker et al., 2002; Barker et al., 2004). I assessed base composition variation at all loci using taxon-specific goodness-of-fit tests (Gruber et al., 2007). Sequence subsets for individual taxa showing significant departures from the overall mean were recoded as appropriate (e.g., AGY coding for mitochondrial DNA third positions, or RY coding for nuclear gene third positions, Phillips and Penny, 2003; Gibson et al., 2005), and potentially affected analyses rerun.

**Integrated Analysis with Published Data:** As noted above, Zhao et al. (2016) recently published an analysis of higher-level certhioid phylogeny. This study used data largely inde-
TABLE 3. Characteristics of gene regions sampled in this study. Shown are the locus names, linkage (based on the Taeniopygia genome), alignment length, numbers of variable and informative characters, and results from single gene analyses using MrBayes v3.2.5 (tree lengths, number of partitions receiving ≥0.95 estimated posterior probability, and mean parameter values under a GTR+I+G parameterization.

| Gene Regions | CYTB | FGB-I4 | FGB-I7 | RAG1 | RAG2 | ZEB1 |
|--------------|------|--------|--------|------|------|------|
| Linkage      | MitochondrialChr 4 | Chr 4 | Chr 5 | Chr 5 | Chr 2 |
| Aligned Length | 1045 | 633 | 1157 | 2876 | 1171 | 1638 |
| Variable Sites (no.) | 470 | 312 | 529 | 690 | 307 | 196 |
| Informative Sites (no.) | 401 | 119 | 200 | 275 | 125 | 65 |
| Tree Length | 2.427 | 0.854 | 0.843 | 0.424 | 0.486 | 0.267 |
| Partitions Supported | 10 | 15 | 19 | 18 | 13 | 11 |
| r_{AC}       | 0.065 | 0.112 | 0.096 | 0.080 | 0.061 | 0.022 |
| r_{AG}       | 0.421 | 0.355 | 0.286 | 0.251 | 0.357 | 0.464 |
| r_{AT}       | 0.064 | 0.070 | 0.058 | 0.048 | 0.034 | 0.038 |
| r_{CG}       | 0.021 | 0.112 | 0.148 | 0.061 | 0.073 | 0.047 |
| r_{CT}       | 0.379 | 0.285 | 0.336 | 0.517 | 0.408 | 0.400 |
| r_{GT}       | 0.049 | 0.066 | 0.076 | 0.043 | 0.067 | 0.030 |
| \pi_A        | 0.314 | 0.307 | 0.324 | 0.312 | 0.288 | 0.335 |
| \pi_C        | 0.403 | 0.174 | 0.164 | 0.214 | 0.218 | 0.235 |
| \pi_G        | 0.099 | 0.181 | 0.190 | 0.244 | 0.238 | 0.186 |
| \pi_T        | 0.184 | 0.338 | 0.322 | 0.230 | 0.256 | 0.245 |
| a            | 1.008 | 8.984 | 8.328 | 0.931 | 1.319 | 1.014 |
| p_i          | 0.625 | 0.078 | 0.109 | 0.383 | 0.406 | 0.702 |

RESULTS

Data Characteristics and Single Gene Analyses: I obtained sequence for all taxa from all loci, though a handful of taxa were incomplete for some loci, including Salpornis miss-
ing the 5’ half of FGB-I4, three species missing 83–133 bases from FGB-I7, and _Nannus_ missing 323 bases from RAG1. All sequences have been submitted to GenBank (see table 1 for accessions), and the concatenated alignment is available at TreeBase (study ID 21870). The alignments obtained from these data ranged in size from 633 (FGB-I4) to 2876 (RAG1) bases in length, with 119 (FGB-I4) to 401 (CYTB) phylogenetically informative sites (table 3). In terms of percentages, ZEB1 yielded the fewest informative sites per sequenced base (4%), the other nuclear loci were approximately equivalent to one another (10%–19%), and CYTB yielded the most (38%). Bayesian phylogenetic analyses of these data sets under a uniform GTR+I+G4 parameterization revealed significant heterogeneity in substitution dynamics among these loci (table 3), as expected given their location in differing genomes (mitochondrial versus nuclear) and variation in coding status (introns versus exons). In particular, based on estimated tree lengths, mitochondrial CYTB evolved at approximately 5× (ranging from 2.8–9.1) the rate of the nuclear genes, and showed much stronger base compositional bias (table 3). In addition to variation among genes, some loci showed variation in base composition among taxa (online supplementary table 1: https://doi.org/10.5531/sd.sp.28). Base frequencies at third positions of _Sitta_ RAG1 deviated significantly from other taxa in the sample (with RAG2 showing a similar, nonsignificant trend), as did third positions of _Dumetella_ CYTB. In the case of CYTB third positions, a number of other taxa showed substantial (but nonsignificant) deviations, primarily in the relative proportion of cytosine and thymine bases, as noted previously for mitochondrial genomes (Gibson et al., 2005; Powell et al., 2013). To assess the impact of these deviations, phylogenetic analyses of data from these genes and taxa were repeated with RY (RAG1) and AGY (CYTB) coding.

**Concatenated Analyses:** Model fitting for partitions of the concatenated-sequence data set identified a strategy with seven partitions: (1) CYTB 3rd positions, (2) CYTB 1st positions, (3) CYTB 2nd+ZEB1 3rd positions, (4) FGB-I4+FGB-I7+RAG2 3rd positions, (5) RAG1 3rd positions+RAG2 1st and 2nd positions, (6) RAG1 1st and 2nd positions, and (7) ZEB1 1st and 2nd positions. Both maximum-likelihood and Bayesian analyses of the data with this model, recoding non-stationary sites as identified above, yielded a single tree (fig. 4). In this instance, data recoding appeared to have little impact on the inferred tree, and the best tree from the original data was nearly identical (not shown), differing only in recovery of _Salpornis_ and _Certhia_ as sister taxa, and bootstrap values were nearly indistinguishable (not shown). Notably, 17 of 31 bipartitions in concatenated analysis received substantial support (defined as ≥0.90 posterior probability) from at least three individual genes (fig. 5). Concatenated support for this tree was generally good, with 18/31 bipartitions receiving ≥75% bootstrap values, and 21/31 receiving ≥0.95 estimated posterior probabilities (plotted on the concatenated tree estimate in fig. 4). Resolution was best for outgroup relationships and relationships among the wrens and allies. In particular, these data supported: monophyly of the Certhioidea relative to the outgroups included here, monophyly of the New World certhioids (Trogloptilidae + Polioptilidae) and of the two families in the clade; and many intergeneric relationships within the Trogloptilidae. By contrast, basal relationships among certhioids were not well resolved by these data, with the strongest support being for separation of _Sitta_ from all other taxa in only 59% of bootstrap replicates of the recoded data (fig. 4).
FIGURE 4. Best estimate of certhioid phylogeny, based on concatenated analysis of 8517 bases from five gene regions (cytb, FGB-I4, FGB-I7, RAG1, RAG2, and ZEB1). Shown is the maximum-likelihood tree from a partitioned RAxML analysis, with nonstationary partitions AGY-recoded (see text). Bootstrap support values (from 1000 fast bootstrap replicates; left) and estimated posterior probabilities from partitioned, concatenated Bayesian analysis (right) are shown below each branch. Node numbers correspond to barplots in figure 5.

AMONG-GENE CONFLICT AND SPECIES TREE ANALYSES: Single-locus analyses indicated several instances of significant conflict over phylogenetic relationships (fig. 5). In four cases (nodes 6, 18, 19, and 22), one (but never more than one) gene region showed strong (≥0.90 posterior probability) conflict with one or more genes supporting that relationship. In an additional four cases (nodes 3, 21, 29, and 30) single genes strongly conflicted with the consensus topology, although no other individual genes strongly supported it. Presumably due to this conflict, only two of these eight nodes (6 and 19) were consistently supported in both likelihood and Bayesian concatenated analyses (figs. 4, 6). Species tree analyses with *BEAST accounting for this among-locus heterogeneity in history yielded a maximum clade credibility tree (fig. 6) congruent with concatenation analyses, with two exceptions. These exceptions were placement of Certhia and
FIGURE 5. Evaluation of support and conflict for individual nodes recovered in concatenated analysis of five gene regions. For each node (see fig. 4 for definitions), a barplot indicates the strength of support for that node found in independent analyses of each gene region using MrBayes with a GTR+I+G parameterization as dark bars above the abscissa, as well as the strength of support against that node (defined as the highest support for all conflicting nodes recovered; light bars below the abscissa). The estimated posterior probabilities from concatenated analysis are shown after each node number.
Salpomis as sister taxa, and placement of Salpinctes as sister to Catherpes+Hylorchilus: neither of these relationships received strong support from either the concatenated or species tree analyses. As expected given the evidential shift from sites to loci, support for certhioid relationships in species tree analyses was generally lower than for concatenated analyses, with 25/31 bipartitions in the species tree receiving higher posterior probabilities in concatenation. However, six bipartitions actually had higher support in species tree analysis, most notably including: monophyly of a clade of wrens excluding Catherpes, Hylorchilus, Microcerculus, Salpinctes, and Odontorchilus, and a sister group relationship between Henicorhina and Cyphorhinus.

Phylogenetic Hypothesis Tests: Specific a priori hypothesis testing was performed for the concatenated data (table 4). All alternative hypotheses except one were very strongly rejected (Kass and Raftery, 1995) by empirical Bayes factor comparisons with the unconstrained analysis. The exception was monophyly of the genus Trogodytes in the broad sense (i.e., including Nannus, Thryorchilus, and Trogodytes), which had a marginal likelihood essentially indistinguishable from the unconstrained analysis.
TABLE 4. Results of constrained Bayesian analyses of the concatenated data. Shown are the stepping-stone (SS) estimates (Xie et al., 2011) of marginal likelihoods from an unconstrained analysis and analyses with several a priori phylogenetic constraints, as well as empirical Bayes factors (twice the natural log of K, 2lnK) comparing constrained to unconstrained likelihoods.

| Hypothesis                                          | SS Marginal Likelihood | 2lnK   |
|-----------------------------------------------------|------------------------|--------|
| Unconstrained                                       | -39897.06              | -0.02  |
| Troglodytes+Thryorchilus+Nannus                     | -39897.07              | -0.02  |
| Troglodytes+Nannus                                  | -39975.19              | -156.26|
| All former Thryothorus                              | -40265.38              | -736.64|
| All former Thryothorus except Thryothorus           | -39922.19              | -50.26 |

INTEGRATED ANALYSIS WITH PUBLISHED DATA: Analysis of the data reported here in conjunction with the previously published data of Zhao et al. (2016) yielded a slightly more resolved picture of basal certhioid relationships (fig. 7). In particular, concatenated analyses strongly supported a sister-group relationship between *Salpornis* and *Certhia*, although this relationship only had a posterior probability of 0.28 in species tree analysis, and cytochrome *b* strongly conflicted with this relationship (fig. 8). As found in analyses of the broader taxon sample with fewer loci (see above), monophyly of Certhioidea was strongly supported (albeit with only two outgroups), as was a sister-group relationship between the wrens (Troglodytidae) and gnatcatchers (Polioptilidae). Relationships among other certhioid lineages (*Sitta* and *Tichodroma*) remained unresolved.

DISCUSSION

Basal Certhioid Relationships

The two previous studies with adequate higher-level sampling of certhioids (Sibley and Ahlquist, 1990; Zhao et al., 2016) were completely congruent in their estimate of relationships in the group (fig. 1). The current study does not significantly contradict previous results, and does little in the way of corroboration. In particular, concatenated analysis of the data reported here fails to recover the previously reported sister-group relationships between *Tichodroma* and *Sitta* and between *Salpornis* and *Certhia*, although the latter relationship was recovered in species tree analyses (with poor support). Perhaps the most notable contribution to resolving certhioid relationships here is an increase in support for the sister-group relationship between *Salpornis+Certhia* and the New World wren/gnatcatcher clade. Although Zhao et al. (2016) recovered this relationship in concatenated analysis, it only received a posterior probability of 0.82, and it was not found in the species tree. In combined analyses of most of Zhao et al.’s data and the new data reported here (fig. 7), this relationship received a posterior probability of 0.99 in concatenated analysis, and was also recovered in the species tree analysis, albeit with an estimated posterior of 0.40. It is likely that phylogenomic approaches (e.g., ultraconserved elements; McCormack et al., 2013) will be necessary to resolve basal relationships of this group with certainty.
Both the gene and taxon sampling of this study are the best to date for addressing higher-level relationships of wrens and gnatcatchers. As expected based on previous results (reviewed above), wrens and gnatcatchers form a well-supported clade within Certhioidea. Within the wrens, there is strong support for a basal split between a small clade of wrens with terrestrial habits (*Microcerculus*, *Salpinctes*, *Catherpes*, and *Hylorchilus*; a total of eight species, termed here the geophilous wrens) and all other wrens. Previous studies (Barker, 2004; Mann et al., 2006) were ambiguous regarding the rooting of the wren tree, with one indel in FGB-I4 (Barker, 2004: indel 6) pointing to a root at *Salpinctes*, a member of the geophilous wren clade. Based on analysis of nucleotide variation in the genes sampled here, it is apparent that that indel was either homoplastic or a misalignment. Reexamination of the alignment shows that the indel involves a simple 11 base pair tandem repeat that has diverged by 1 base pair in *Salpinctes*, driving its alignment against the second repeat unit that is identical to *Salpinctes* in an outgroup, rather than alignment with other wrens.

Perhaps the only outstanding question regarding basal relationships of wrens is the placement of the South American endemic genus *Odontorchilus*. This involves one of only four cases of hard incongruence among genes in this data set (fig. 5: node 18). Two genes (FGB-I4 and RAG2) strongly support placement of the genus outside the main radiation of nongeophilous
wrens, whereas one gene (FGB-I7) strongly supports its placement as sister to one of the two main clades in this radiation. A third gene (RAG1) is congruent with RAG2, but with support just below 0.9 posterior probability. *Odontorchilus* wrens have long been recognized as distinct, in particular due to their toothed bill (for which they are named), their preferred foraging stratum in the canopy, and their simple trilled songs; consequently, no there is no clear a priori expectation for their placement anywhere within wrens. However, it is interesting to find a South American lineage placed so deeply within the family (see below).

**Relationships of Nongeophilous Wrens**

The nongeophilous wrens apart from *Odontorchilus* are divided into two well-supported clades (fig. 4: A, B) of nearly the same number of genera (seven and eight, respectively), and species (42 and 36, respectively). Clade A comprises subtropical and tropical species best known for their singing ability and nearly ubiquitous habit of performing vocal duets (Mann et al., 2009). Clade B comprises genera with both tropical and temperate distributions, including three small genera (two monotypic) with only temperate species. Many more species in this clade do not perform vocal duets, and species in two genera nest in tree cavities, a behavior otherwise only known from the genus *Microcerculus*, which nest in tunnels in the soil.

Clade A includes three genera formerly subsumed in the genus *Thryothorus*, until molecular data strongly supported placement of the type of that genus (the Carolina Wren *T. ludovicianus*) as sister to *Thryomanes*, to the exclusion of all other species that had been placed in it (Barker, 2004; Mann et al., 2006). Mann et al. (2006) recognized three clades of former “*Thryothorus*” wrens as genera (*Pheugopedius, Thryophilus*, and *Cantorchilus*), giving a new name to the third. This treatment of these taxa is strongly corroborated by the current study. In particular, Bayes factor comparisons strongly reject association of *Thryothorus ludovicianus* with the other former members of the genus, as expected given previous analyses. In addition, Bayes factors strongly reject a monophyletic origin of the three genera in which former *Thryothorus* species are now placed, suggesting that these species cannot be subsumed under the oldest generic name (*Pheugopedius*) for the sake of simplicity alone. Relationships within this clade are generally strongly supported in concatenated Bayesian but not in concatenated-likelihood or species tree analyses (figs. 4, 6). Notably, the genus *Pheugopedius* is strongly supported (except in species tree analysis) as sister to all other genera in the group. In concatenated Bayesian analyses, *Cantorchilus* is strongly supported as sister to *Cinnycerthia*, and *Thryophilus* as sister to *Uropsila*, explaining why monophyly of former *Thryothorus* species is strongly contradicted by these data. Relationships of *Henicorhina* and *Cyphorhinus* are less clear, with maximum-likelihood analysis (fig. 4) favoring them as sequential sister taxa to *Uropsila+Thryophilus*, and species tree analysis (fig. 6) placing them as sister taxa (neither relationship with appreciable support).

Relationships among Clade B wrens were better resolved than those in Clade A. Four of six intraclade relationships were strongly supported by both concatenated and species tree analyses (figs. 4, 6). These well-supported relationships included the previously recovered sister-group relationship between the monotypic genera *Thryothorus* and *Thryomanes*, and their sister-group
relationship to the diverse (both phenotypically and in species numbers) genus Campylorhynchus. The remaining four genera of Clade B formed a well-supported sister clade to these three. The only strongly supported relationship among these four genera placed the genus Troglodytes (a diverse, cosmopolitan, but relatively morphologically uniform group) sister to Thryorchilus (a distinct monotypic genus of the Central American highlands). It is possible that this close relationship is actually due to Thryorchilus falling within Troglodytes, although the one study with broader sampling of the latter suggests otherwise (Gómez et al., 2005). The remaining ambiguities lie in the relative placement of the genera Nannus, Cistothorus, and Ferminia.

The genus Nannus is the only lineage of the wren and gnatcatcher clade with species in the Old World, comprising at least three species Holarctic in distribution (Drovetski et al., 2004). Until recently, these birds were classified as a single species in the genus Troglodytes: the Winter Wren, T. troglodytes. Mitochondrial studies have consistently pointed
toward a distant relationship of Nannus species to core Troglodytes, with Thryorchilus and possibly Cistothorus intervening between the two (Rice et al., 1999; Gómez et al., 2005). In the current study, both Cistothorus and the genus Ferminia (never before included in a molecular phylogeny) separate Nannus from Troglodytes, though neither relationship showed substantial support (figs. 4, 6). Bayes factor comparison of these results to an analysis with Troglodytes monophyly constrained strongly favored the former (table 4). However, the marginal likelihood of an analysis with Nannus, Thryorchilus, and Troglodytes constrained as monophyletic was indistinguishable from the unconstrained analysis (table 4), indicating that the strongest signal is for monophyly of Troglodytes+Thryorchilus. Thus, these data would not contradict a classification that subsumed all three genera (Nannus, Thryorchilus, and Troglodytes) within Troglodytes, as previously done by its describer (Bangs, 1902), and some subsequent taxonomies (e.g., Paynter and Vaurie, 1960). Unless subsequent data strongly separate Nannus from Troglodytes+Thryorchilus, this would seem appropriate, despite the distinctiveness of Thryorchilus.

The remaining two lineages in this unresolved region of the wren tree are Cistothorus and Ferminia. The former genus comprises at least five species distributed from northern North America through the cape region of South America (including the Islas Malvinas). All Cistothorus species are associated with grasslands and marsh habitats, and one widespread species (C. platensis) is found in grasslands from Central America nearly to Cape Horn (though this species is in need of splitting; Robbins and Nyári, 2014). The genus Ferminia is monotypic, with the single species F. cerverai endemic to Cuba, where it is restricted to the Zapata swamp of southern Matanzas province (Garrido and Kirkconnell, 2000). The species exhibits some interesting parallels with Cistothorus, including living in a marsh habitat, construction of woven domed nests on grasses or emergent vegetation (Martínez and Martínez, 1991; Llanes Sosa and Mancina, 2002; Forneris and Martínez, 2003), and vocal similarities. Based on recordings and videos (Internet Bird Collection, http://www.hbw.com/ibc/species/zapata-wren-ferminia-cerverai; Xeno-canto, http://www.xeno-canto.org/species/Ferminia-cerverai; both accessed September 2017), male Ferminia have vocal repertoires (e.g., see Xeno-canto catalog XC256894), as in many wren species (e.g., Kroodsma, 1975; Kroodsma and Verner, 1978; Molles and Vehrencamp, 1999; Logue, 2006; Bradley and Mennill, 2009), and may engage in matched countersinging as seen in some Cistothorus (Kroodsma and Verner, 1978). At least some Ferminia songs include a series of repeated low-frequency syllables most closely matched among wrens, based on my extensive listening to wren vocalizations both in the field and in recordings, by songs of the marsh wren C. palustris (e.g., fig. 9). In terms of plumage, it is perhaps most similar to Troglodytes, with dull brownish-dun underparts and richer brown strongly barred upperparts (first figured by Brooks in Barbour, 1928, or see Forneris and Martínez, 2003, for a photograph), a similarity noted but dismissed by Barbour in his description of the species (Barbour, 1926). Consequently, it is perhaps unsurprising to find this species in an indeterminate position relative to these two genera. Regardless of how these relationships are ultimately resolved, this mosaic pattern of phenotypic similarities and closely spaced divergences suggests relatively rapid diversification at the base of this group.
FIGURE 9. Comparison of song characteristics between *Ferminia cerverai* and *Cistothorus palustris*. Shown is the spectrogram of a single *Ferminia* song (seconds 1.93–4.28 of Xeno-Canto catalog number XC256894, recorded by Hans Matheve and reproduced with permission), compared to the spectrogram of a single *C. palustris* song (seconds 1.77–2.92 of Xeno-Canto catalog number XC386079, recorded by the author) with a similar repeated syllable. Both spectrograms were generated using the “spectro” function of the R package “Seewave” (Sueur et al., 2008; the *C. palustris* recording was also filtered from 0–1.1 kHz to remove low-frequency background noise).

**Biogeography and Diversification of Certhioidea**

Although not well resolved phylogenetically, the earliest divergences within Certhioidea are among Old World or ancestrally Old World lineages (fig. 10). Both *Tichodroma* and *Salpornis* are exclusively Old World, and both *Certhia* (Tietze et al., 2006) and *Sitta* (Pasquet et al., 2014) clearly have Old World origins, despite each lineage having invaded the New World (once in *Certhia* and three times in *Sitta*). Thus, certhioids are undoubtedly Old World (possibly north temperate) in origin, consistent with an Old World origin for the entire certhioid-muscicapoid clade and indeed for oscines as a whole (Barker et al., 2004; Moyle et al., 2016). While dispersal of *Certhia* and *Sitta* into the New World has not resulted in substantial diversification (a total of six species by current taxonomy, but possibly as many as 10; Manthey et al., 2011; Walstrom et al., 2012), another certhioid lineage—the ancestor of the wrens and gnatcatchers—dispersed into the New World and diversified both in species number (a total of 106 species; Gill and Donsker, 2017) and in ecologically related phenotypic traits such as body size, limb proportions, and beak shape.
Based on a secondary point calibration (Moyle et al., 2016), the New World Certhioidea (NWC) most likely dispersed from the Old World between 16.1 (stem age) and 11.8 million (crown age) years ago, the mid-Miocene (fig. 10; this represents a minimum range, since no uncertainty was included in scaling of this tree). If this range of estimated dispersal times is accurate, it suggests a north Pacific route into the New World via Beringia, rather than a North Atlantic or Antarctic route (Sanmartin et al., 2001). No extant member of the NWC is a strong flier, and long-distance dispersal directly into South America seems unlikely. Consequently, it is likely that the NWC diversified in North America prior to independent invasion of South America by multiple lineages, as has been previously suggested for wrens (Mayr, 1946), and as seems the case for emberizoids (Barker et al., 2015). Within wrens, this is corroborated by the
primarily North American distribution of the geophilous wrens (half of the first split in the Troglodytidae); however, the relatively deep placement of the South American Odontorchilus suggests early dispersal into South America (or possibly extinction of this lineage from the north; fig. 10). By contrast, all three genera of Poliopitilidae are found in both North and South America: if possible, resolution of the ancestral area of this clade will require species-level as well as (most likely) extensive intraspecific sampling.

Assuming a northern origin, a minimum of six dispersal events into South America are required to explain the extant distribution of wrens, including dispersals of Microcerculus, Odontorchilus, Clade A, Campylorhynchus, Cistothorus, and Troglodytes (fig. 10). In some cases, extant distributions would most likely require additional dispersals south or back dispersals from the south to the north: current taxon sampling does not merit a formal analysis. Nevertheless, it is clear that the NWC has had a long history in both North and South America. In particular, the origin of Clade A by dispersal into South America would imply presence of wrens in the Pliocene or earlier, supporting either overwater dispersal of this lineage (not unreasonable given the presence of Troglodytes wrens on islands as remote as Isla Clarion and Isla Socorro, although it is worth noting that species in this genus are migratory), or early closure of the isthmus, as has recently been hypothesized (Bacon et al., 2015; Montes et al., 2015; but see O’Dea et al., 2016). Complete taxon sampling of this lineage may clarify this history, in particular the timing and directional bias of dispersal events and subsequent diversification rates (e.g., Barker et al., 2013; Barker et al., 2015).

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## APPENDIX 1

**Supplementary Primers Used in Amplification of *Ferminia* Sequences**

| Gene | Primer Name         | Sequence                              | Bases | Direction |
|------|---------------------|---------------------------------------|-------|-----------|
| CYTB | L15068-Ferminia     | CTAGCTATACACTATAACAGCAGA              | 23    | forward   |
| CYTB | L15410-Ferminia     | TGGGGGCCCTTTTGCAGTACACAA              | 23    | forward   |
| CYTB | L15848-Ferminia     | CTAACAGCTCAATGACCTTCCG                | 25    | forward   |
| CYTB | H15103-Ferminia     | TCAGCCAAACTGGACGTTTCGGCA              | 24    | reverse   |
| CYTB | H15460-Ferminia     | GTGGACTAGTGTATGCTCCTGGGAT             | 24    | reverse   |
| CYTB | H15709-Ferminia     | GCGATAGCAAAATGAGAAGATAC              | 23    | reverse   |
| CYTB | H15934-Ferminia     | GCCTAGTTTGCTCCGATGATAATGAA           | 24    | reverse   |
| FGB-I4| FGBI4-243F          | CAGGCTTTTTGTGAGGTGGA                 | 20    | forward   |
| FGB-I4| FGBI4-419R          | TGGTGCAGCTTTTCCAAAGAGG               | 21    | reverse   |
| FGB-I7| FGBI7-754F          | ACCGTCTAGCAAACATCCTTG                | 20    | forward   |
| FGB-I7| FGBI7-565F          | AGGAATTTTATGTTGTCGGGT               | 21    | forward   |
| FGB-I7| FGBI7-359F          | TATGACTGACAGCACAGCTG                 | 20    | forward   |
| FGB-I7| FGBI7-164F          | ATCCCTGCAAAGTTACCAGCT                | 20    | forward   |
| FGB-I7| FGBI7-852R          | ACCTGCCCATGACTGAAAT                  | 20    | reverse   |
| FGB-I7| FGBI7-628R          | TCAAGGAATATCCTAAAGATAGGAAAGCA       | 26    | reverse   |
| FGB-I7| FGBI7-446R          | GCCTCTTTTCTCCAGGACCA                 | 20    | reverse   |
| FGB-I7| FGBI7-324R          | TTGCCTCTTCTTCCAGTGGTA               | 20    | reverse   |
| FGB-I7| FGBI7-277R          | GTGTGCTTTCTTCTGGCTTG                 | 20    | reverse   |
| RAG1 | RAG1-2770F          | CTGGGCCGAGGAGGAGGGAATG               | 20    | forward   |
| RAG1 | RAG1-2279F          | GATGCATTGCACTGCGACAT                 | 20    | forward   |
| RAG1 | RAG1-2185F          | GAGGTCCACCCCATACCAAGC                | 20    | forward   |
| RAG1 | RAG1-2106F          | GCTTGGAGGCATCCGAGAT                  | 20    | forward   |
| RAG1 | RAG1-1747F          | TGTCAGGAGAAAGCGGTTC                  | 20    | forward   |
| RAG1 | RAG1-1523F          | GGACTGCCACACTCAATCGA                 | 20    | forward   |
| RAG1 | RAG1-1456F          | TGAGTGCAAGACCTCCCTGCA                | 20    | forward   |
| RAG1 | RAG1-1273F          | AGGGAGGGGAGATCTGAGCTTC              | 20    | forward   |
| RAG1 | RAG1-1273F          | AGGGAGGGGAGATCTGAGCTTC              | 20    | forward   |
| RAG1 | RAG1-1493F          | CACGACAAATGAGGGTTGCCAGC              | 20    | forward   |
| RAG1 | RAG1-1269F          | AGAACCTTACCCCAATGCGAGG               | 20    | forward   |
| RAG1 | RAG1-106F           | TGAAGCAGTGCAGGAGGAGG                 | 20    | forward   |
| RAG1 | RAG1-2660R          | GCCTCGCAAAACGCTGTGAA                | 20    | reverse   |
| RAG1 | RAG1-2298R          | ATGTCGACAGTGCATGCAAT                 | 20    | reverse   |
| RAG1 | RAG1-2206R          | CTGCTGGAAGGAGTGGTGAGG                | 20    | reverse   |
| RAG1 | RAG1-2125R          | ATCTGTGGAATGTCCCAAGAC               | 20    | reverse   |
| RAG1 | RAG1-1766R          | GAACGCGCTTCTCTGGGACA                 | 20    | reverse   |
| RAG1 | RAG1-1541R          | CGATTGAGTGCTGGCCAGCTC               | 20    | reverse   |
| Gene  | Primer Name   | Sequence                      | Bases | Direction |
|-------|---------------|-------------------------------|-------|-----------|
| RAG1  | RAG1-1292R    | GAAGTCCAGATCCCCCTCCCT        | 20    | reverse   |
| RAG1  | RAG1-892R     | AGGATACCGACAGGGGG            | 19    | reverse   |
| RAG1  | RAG1-600R     | CGTTGCCACCTTGTAACACT         | 20    | reverse   |
| RAG1  | RAG1-R16      | GTTGGGGAGTGGGTTGCA           | 21    | reverse   |
| RAG1  | RAG1-397R     | GACATCCCTCCGACATCAA          | 20    | reverse   |
| RAG1  | RAG1-356R     | GATCTGGCCAAGAGTTGCT          | 20    | reverse   |
| RAG1  | RAG1-125R     | CTCCCTTTGGACTGCTCCTCA        | 20    | reverse   |
| RAG2  | RAG2-881F     | AGGGACATCTCCACCAAGGT         | 20    | forward   |
| RAG2  | RAG2-750F     | CAGTCGACTACGCTTCCA           | 20    | forward   |
| RAG2  | RAG2-610F     | GAAGTGAGTGGCCCTCCCAAG        | 20    | forward   |
| RAG2  | RAG2-412F     | CATTGCTTTGCATGCCAGC          | 20    | forward   |
| RAG2  | RAG2-216F     | TCCTTTCTGCTCTCTTTGCA         | 20    | forward   |
| RAG2  | RAG2-1033R    | GCAAGGGCTGATGAGTACCA         | 20    | reverse   |
| RAG2  | RAG2-895R     | GTGGAGATGGTGCCCTGGAAAG       | 20    | reverse   |
| RAG2  | RAG2-692R     | TCCAGAGCTTCAAGACGGAC         | 20    | reverse   |
| RAG2  | RAG2-496R     | GTGCCGAGATGAGTACCTAGC        | 21    | reverse   |
| RAG2  | RAG2-306R     | CTGTTTTGCTTGGCCATTTCA        | 20    | reverse   |
| ZEB1  | ZEB1-1246F    | AACACATCTCGATCACCAG          | 20    | forward   |
| ZEB1  | ZEB1-854F     | CCCCTGCCCACATCAGAAA          | 20    | forward   |
| ZEB1  | ZEB1-393F     | AGTGCCAGTGATGGTAAGC          | 20    | forward   |
| ZEB1  | ZEB1-296F     | GTTCTCCTCAGGGTGTTGG          | 20    | forward   |
| ZEB1  | ZEB1-57F      | GTCAGGGCTCAAGATGTTGCT        | 20    | forward   |
| ZEB1  | ZEB1-1618R    | AGCAAGGGGAACTGTTCTG          | 20    | reverse   |
| ZEB1  | ZEB1-1538R    | GGATTGGCACTTGGTGGA           | 19    | reverse   |
| ZEB1  | ZEB1-1161R    | GCTGGTCTCGAGGTTCCGCT         | 20    | reverse   |
| ZEB1  | ZEB1-856R     | GGGACCTCAGGTTTCTGCAGC        | 20    | reverse   |
| ZEB1  | ZEB1-819R     | GGGAAAGCTGAGAAGGGCTTT        | 20    | reverse   |
| ZEB1  | ZEB1-412R     | CGTTACCATCACCAGCCACT         | 20    | reverse   |
| ZEB1  | ZEB1-315R     | CACCCACCTGGAGGAAAC           | 20    | reverse   |
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