



RESEARCH ARTICLE

## Willet be one species or two? A genomic view of the evolutionary history of *Tringa semipalmata*

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### ABSTRACT

The Willet (*Tringa semipalmata*; Scolopacidae) is composed of 2 morphologically and vocally distinct subspecies with broadly disjunct breeding distributions in North America. Nominate *T. s. semipalmata* breeds in coastal salt and brackish marshes along the Atlantic and Gulf coasts of eastern North America and the West Indies, and *T. s. inornata* breeds in wet grasslands and prairies in the northwestern interior of North America. To assess divergence and test for hybridization between the 2 subspecies, we sampled breeding and wintering populations and collected morphological data, mitochondrial DNA sequences from the ND2 locus, and nuclear DNA sequences from genomic libraries enriched for ultraconserved elements (UCEs). Mitochondrial haplotypes were reciprocally monophyletic between the 2 subspecies and indicated divergence approximately 700,000 yr ago. The UCE dataset included 4,635 loci containing 19,322 single nucleotide polymorphisms (SNPs), and, based on these data, individuals clustered by subspecies with no evidence of admixture between them and no substructure within subspecies. We identified 42 nuclear loci that contained SNPs fixed for alternate alleles between the 2 subspecies. Of the 42 loci with fixed differences, a statistically disproportional 17 were Z-linked, indicating a role for sexual selection in the divergence of the 2 subspecies. Genetic, morphological, ecological, and behavioral differences suggest that the 2 Willet subspecies may merit treatment as separate species. Further studies are needed to determine the presence of pre- or post-mating reproductive isolation.

**Keywords:** ultraconserved elements, shorebirds, *Tringa*, Z chromosome, species delimitation, sexual selection, drift, systematics

### ¿*Tringa semipalmata* es una especie o dos? Una mirada genómica de su historia evolutiva

#### RESUMEN

*Tringa semipalmata* (Scolopacidae) es una especie compuesta por dos subespecies morfológicamente y vocalmente distintas, con distribuciones reproductivas en gran medida disjuntas en América del Norte. *T. s. semipalmata* cría en pantanos salobres costeros a lo largo del Atlántico y del Golfo en el este de América del Norte y de las Indias Occidentales, y *T. s. inornata* cría en los pastizales húmedos y las praderas en el interior al noroeste de América del Norte. Para estimar la divergencia y evaluar una hibridación entre las dos subespecies, muestreamos las poblaciones reproductivas e invernales y colectamos datos morfológicos, secuencias de ADN mitocondrial del locus ND2 y secuencias de ADN nuclear de bibliotecas genómicas enriquecidas con elementos ultra-conservados (UCE por sus siglas en inglés). Los haplotipos mitocondriales fueron recíprocamente monofiléticos entre las dos subespecies e indicaron una divergencia hace aproximadamente 700,000 años. La base de datos de UCE incluyó 4,635 loci conteniendo 19,322 polimorfismos de nucleótido único (SNP por sus siglas en inglés), y basados en estos datos los individuos se agruparon por subespecies sin que haya evidencia de mezcla entre ellos y ni sub-estructura al interior de las subespecies. Identificamos 42 loci nucleares que contuvieron SNPs fijos para alelos alternos entre las dos subespecies. De los 42 loci con diferencias fijas, una cantidad estadísticamente desproporcionada de 17 estuvieron vinculados al Z, indicando que la selección sexual juega un rol en la divergencia de las dos subespecies. Las diferencias genéticas, morfológicas, ecológicas y comportamentales sugieren que las dos subespecies de *Tringa semipalmata* pueden merecer un tratamiento como especies separadas. Se necesitan estudios adicionales para determinar la presencia de aislamiento pre- o post-apareamiento reproductivo.

**Palabras clave:** aves playeras, cromosoma Z, delimitación de especies, deriva, elementos ultra-conservados, selección sexual, sistemática, *Tringa*

## INTRODUCTION

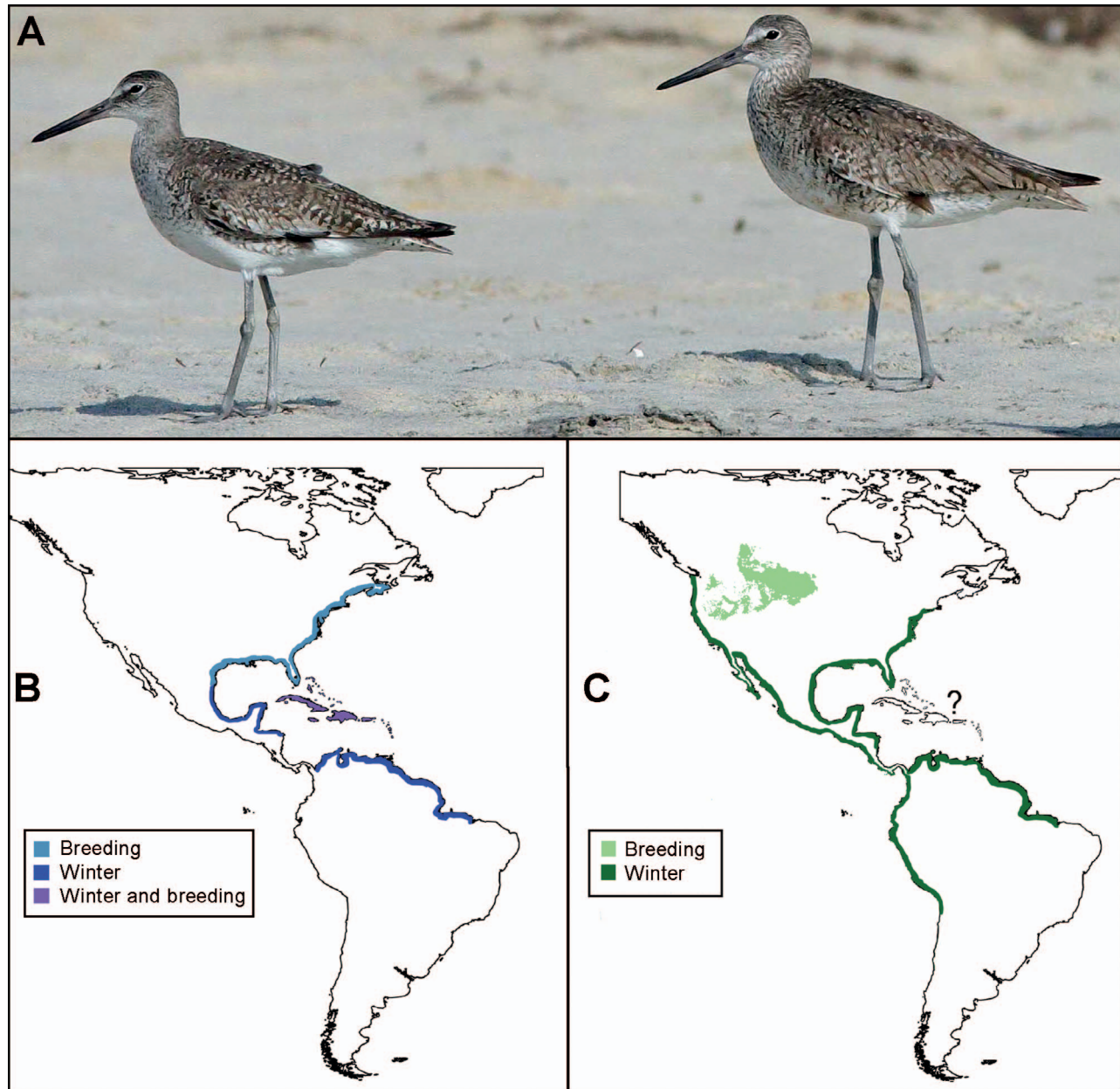
Recent advances in our understanding of behavioral, ecological, and genetic diversity in North American birds have resulted in the recognition of greater biological species diversity, for example in Gunnison (*Centrocercus minimus*) and Greater sage-grouse (*C. urophasianus*; Hupp and Braun 1991, Young et al. 1994, Oyler-McCance et al. 1999, 2015), large *Rallus* species (Maley and Brumfield 2013), Winter (*Troglodytes hiemalis*) and Pacific wrens (*T. pacificus*; Toews and Irwin 2008), Bell's (*Artemisiospiza belli*) and Sagebrush sparrows (*A. nevadensis*; Cicero and Koo 2012), Dusky (*Dendragapus obscurus*) and Sooty grouse (*D. fuliginosus*; Barrowclough et al. 2004), Western (*Aphelocoma californica*) and Island scrub-jays (*A. insularis*; Delaney et al. 2008), and Eastern (*Antrostomus vociferus*) and Mexican whip-poor-wills (*A. arizonae*; Han et al. 2010). In addition to morphological, behavioral, and ecological data, genetic data have been instrumental in illustrating the distinctness and evolutionary independence of these species, but most studies have been restricted to examining a limited number of genetic markers. Studies relying on a few markers are subject to biased inferences of population structure or species limits if the histories of the markers are not representative of the entire genome or do not accurately represent the population's history (Edwards and Beerli 2000). Recent advances in sequencing technology make it possible to obtain markers from loci across the genome, providing a large number of independent samples from which to estimate population history. Genome-wide studies of closely related avian taxa have revealed deep, previously undetected divergences (Maley and Brumfield 2013, Smith et al. 2014, Harvey and Brumfield 2015, Oyler-McCance et al. 2015), genomic islands of elevated divergence (Ellegren et al. 2012), and elevated rates of divergence on the avian Z chromosome (Lavretsky et al. 2015, Dhami et al. 2016).

The Willet (*Tringa semipalmata*) is a widespread, migratory New World shorebird. It is composed of 2 broadly disjunct allopatric breeding populations found across North America and the West Indies that exhibit ecological, vocal, behavioral, and morphological differences (Figure 1A). The eastern subspecies (*T. s. semipalmata* Gmelin 1789; henceforth, *semipalmata*) breeds almost exclusively in salt and brackish marshes along the Atlantic Coast and the Gulf of Mexico south to Tamaulipas, Mexico, and locally in the Caribbean (Howe 1982, Lowther et al. 2001, O'Brien et al. 2006a). The nonbreeding distribution of *semipalmata* needs further study, but it likely includes coastal areas south of the breeding areas, from the Caribbean to eastern South America south to Argentina (Figure 1B; AOU 1957, Howe 1982, Lowther et al. 2001, O'Brien et al. 2006a, Martinez-

Curci et al. 2014, Van Gils and Wiesma 2014). The western subspecies (*T. s. inornata* Brewster 1887; henceforth, *inornata*) breeds in brackish and freshwater wetlands and in grassland habitats in the Great Basin and prairies of the northwestern U.S. and southern Canada (Lowther et al. 2001, O'Brien et al. 2006b). This subspecies winters along rocky shorelines and on beaches along the Pacific Coast from Washington in the U.S. to central Chile, and in coastal areas on the Atlantic Coast from New Jersey south to the Gulf Coast of the U.S., and in northern and eastern South America and occasionally to Argentina (Figure 1C; AOU 1957, Lowther et al. 2001, O'Brien et al. 2006b, Martinez-Curci et al. 2014). The 2 subspecies can be syntopic during migration and on wintering grounds. Further, some *inornata* may remain on their wintering grounds and then are syntopic with breeding *semipalmata* (e.g., in Louisiana, USA; D. Dittman and S. Cardiff personal observation; specimen LSUMZ 71709). Pair bonding in *semipalmata* is known to occur on the breeding grounds (Howe 1982) and, although to the best of our knowledge this has not been studied, is also likely to occur on the breeding grounds in *inornata*. Tomkins (1955) reported that some *semipalmata* arrive on the breeding grounds already paired, but this may simply reflect pair bonding elsewhere within the breeding range and then movement to nest sites, or nest site philopatry. The *inornata* that 'summer' on their wintering grounds are not reproductively active. For example, in Louisiana, USA, summering *inornata* do not attain full prealternate (breeding) plumage or are molting to basic plumage during the breeding period of *semipalmata*, and their gonads do not show evidence of breeding (e.g., specimen LSUMZ 71709; D. Dittman and S. Cardiff personal observation).

Differences in morphological (mensural and plumage) characteristics led Brewster (1887) to describe *inornata* as distinct from *semipalmata*. *T. s. inornata* is larger overall (on average, 10% larger), with a longer wing and tarsus (Pyle 2008). Bill measurements overlap, but *inornata* generally has a longer and broader bill (Pyle 2008). In prealternate plumage, *semipalmata* is darker overall and more heavily patterned. Nondefinitive or transitional plumages are similar. The songs of the 2 subspecies also differ, with the songs of *semipalmata* being higher in frequency and shorter in duration than those of *inornata* (Douglas 1996, 1998), a difference thought to reflect differences in the background acoustic environments between coastal and inland habitats (Douglas 1999).

Douglas (1998) used playback experiments in a breeding population of *semipalmata* to assess whether song could be a premating reproductive isolating mechanism between the 2 subspecies. He found that *semipalmata* (he was unable to distinguish sexes) responded to male 'pill-will-



**FIGURE 1.** (A) Photograph of *Tringa semipalmata semipalmata* (left) and *T. s. inornata* (right) in late July, 2015, at Nags Head, North Carolina, USA. Both individuals are adults in worn alternate plumage. Photo credit: Michael O'Brien; (B) Map of the breeding, wintering, and year-round distribution of *T. s. semipalmata*; and (C) Breeding and wintering distribution of *T. s. inornata*.

willet' songs of *semipalmata*, but not of *inornata*. Because the 'pill-will-willet' song is used during pair bonding (Vogt 1937, Hansen 1979, Sordahl 1979, Howe 1982), this suggests that the song could be a premating reproductive isolating mechanism. However, *semipalmata* did not discriminate between the 'kik' and 'dik' calls that are associated with reproductive behavior (Douglas 1998). Reciprocal playback studies across the distribution of both subspecies are needed to assess whether songs and calls are premating reproductive isolating mechanisms.

Here, we use ultraconserved elements (UCEs), mitochondrial DNA sequence data, and morphological data to characterize genetic and morphological differences between the 2 subspecies and to look for evidence of introgression. UCEs are regions of the genome that are highly conserved in sequence similarity among species (Bejerano et al. 2004). The variable flanking regions of the UCEs allow the assessment of genetic differentiation within and across species (Smith et al. 2013, Harvey and Brumfield 2015, Harvey et al. 2015).



**TABLE 1.** Sample information for individuals of *Tringa semipalmata semipalmata* and *T. s. inornata* sampled in Wyoming, California, Florida (*inornata*), New York, Texas (*semipalmata*), and Louisiana, USA (both subspecies). For each individual in our dataset the following information is provided: the museum where the skin or skeleton specimen resides; skin or skeleton catalog number; tissue number; subspecific identification; collection locality within the U.S.; date of collection; sex identified from gonads; condition: breeding (B), nonbreeding (NB), wintering (W), and migrating (M); and, if applicable, GenBank accession number for ND2 sequence data. An asterisk next to the catalog number signifies that the specimen is a skeleton. LSUMZ is the Louisiana State University Museum of Natural Science, SBCM is the San Bernardino County Museum, FLMNH is the Florida Museum of Natural History, UWYMV is the University of Wyoming Museum of Vertebrates, and AMNH is the American Museum of Natural History.

Museum	Catalog no.	Tissue no.	Subspecies	Collection locality	Collection date	Sex	Condition	GenBank no.
LSUMZ	156047	16877	<i>inornata</i>	California	April 16, 1991	Male	M	KU854980
LSUMZ	156049	16879	<i>inornata</i>	California	April 16, 1991	Male	M	KU854977
LSUMZ	156050	16880	<i>inornata</i>	California	April 16, 1991	Female	M	
LSUMZ	NA	9786	<i>inornata</i>	California	December 1, 1984	Unknown	W	KU854976
LSUMZ	NA	9837	<i>inornata</i>	California	December 1, 1984	Unknown	W	KU854981
SBCM	54276	23154	<i>inornata</i>	California	April 18, 1994	Male	M	
SBCM	54880	24764	<i>inornata</i>	California	August 8, 1996	Male	M	KU854982
LSUMZ	161029*	27058	<i>inornata</i>	California	January 13, 1993	Male	W	KU854978
SBCM	59169	53202	<i>inornata</i>	California	August 24, 2006	Female	M	
FLMNH	44900	59289	<i>inornata</i>	Florida	August 17, 2005	Female	W	
LSUMZ	159110	19407	<i>inornata</i>	Louisiana	April 25, 1993	Female	M	KU854979
LSUMZ	185324	71705	<i>inornata</i>	Louisiana	March 27, 2011	Female	W/M	
LSUMZ	185328	71709	<i>inornata</i>	Louisiana	June 16, 2011	Female	NB	KU854975
UWYMV	2808	747	<i>inornata</i>	Wyoming	July 9, 2014	Male	B	
UWYMV	2809	748	<i>inornata</i>	Wyoming	July 9, 2014	Female	B	
UWYMV	2810	749	<i>inornata</i>	Wyoming	July 9, 2014	Female	B	
UWYMV	2812	782	<i>inornata</i>	Wyoming	July 9, 2014	Female	B	
UWYMV	2813	783	<i>inornata</i>	Wyoming	July 9, 2014	Male	B	
UWYMV	2811	784	<i>inornata</i>	Wyoming	July 9, 2014	Male	B	
AMNH	26045*	5985	<i>semipalmata</i>	New York	June 1, 1999	Male	B	KU854965
AMNH	26044*	5966	<i>semipalmata</i>	New York	May 1, 2000	Female	B	KU854969
AMNH	26046*	5967	<i>semipalmata</i>	New York	June 1, 2000	Female	B	KU854973
LSUMZ	151993	15556	<i>semipalmata</i>	Louisiana	March 18, 1990	Male	M/B	KU854966
LSUMZ	173598	43221	<i>semipalmata</i>	Louisiana	May 31, 1999	Male	B	KU854967
LSUMZ	183976	61138	<i>semipalmata</i>	Louisiana	April 15, 2007	Male	B	
LSUMZ	185134	62980	<i>semipalmata</i>	Louisiana	June 15, 2010	Unknown	NB (chick)	KU854968
LSUMZ	185325	71706	<i>semipalmata</i>	Louisiana	March 27, 2011	Female	B	KU854971
LSUMZ	185326	71707	<i>semipalmata</i>	Louisiana	March 27, 2011	Male	B	KU854972
LSUMZ	185327	71708	<i>semipalmata</i>	Louisiana	June 16, 2011	Female	B	KU854974
LSUMZ	175755	47313	<i>semipalmata</i>	Texas	May 5, 2001	Male	B	KU854970

## METHODS

We sampled 30 *Tringa semipalmata* individuals (19 *inornata*, 11 *semipalmata*; Table 1). Our sample of *inornata* included 6 individuals from 1 breeding population (Wyoming, USA), with the rest representing nonbreeding individuals from California, Louisiana, and Florida, USA. For *semipalmata*, we sampled individuals from the Atlantic (New York, USA) and Gulf Coast (Louisiana and Texas, USA) breeding populations, but lacked samples from any Caribbean breeding population. We inferred breeding condition from gonad size and plumage traits (Table 1). Wintering individuals were identified to subspecies based on morphology.

We extracted total DNA from the pectoral muscle using a DNeasy tissue extraction kit (Qiagen, Valencia, California, USA). After quantification using a Qubit 2.0 (ThermoFisher Scientific, Waltham, Massachusetts, USA), we sent 2

µg of DNA at a concentration of 40–100 ng µl<sup>-1</sup> to Rapid Genomics (Gainesville, Florida, USA) for sequence capture using the Tetrapods-UCE-K5v1 probe set (ultraconserved. org) and sequencing following the protocol outlined by Faircloth et al. (2012). Samples were multiplexed at 160 samples per lane on a 100 base pairs (bp) paired-end Illumina HiSeq 2500 run (Illumina, San Diego, California, USA).

## Bioinformatics

We demultiplexed raw reads using Casava 1.8 (Illumina) and cleaned reads with Illumiprocessor (Faircloth 2013). We used the seqcap\_pop pipeline ([https://github.com/mgharvey/seqcap\\_pop](https://github.com/mgharvey/seqcap_pop)) to process the assembled datasets. We used Velvet (Zerbino and Birney 2008) and the wrapper program Velvet Optimiser (<http://bioinformatics.net.au/software/velvetoptimiser.shtml>), exploring hash lengths of between 67 and 71, to assemble reads across

all individuals into contigs (contiguous sequences of DNA created by assembling overlapping sequenced fragments of a chromosome) de novo. We mapped contigs to UCE probe sequences using Phyluce (Faircloth 2015). For each individual, we mapped reads to contigs that mapped to UCEs using the program bwa (Li and Durbin 2009). We explored thresholds that allowed anywhere from 1 to 7 mismatches between reads for mapping. We converted sam files to bam format using SAMtools (Li et al. 2009), and cleaned bam files by soft-clipping reads outside the reference contigs with Picard (<http://broadinstitute.github.io/picard/>). We added read groups for each individual using Picard and merged the bam files across individuals with SAMtools. We realigned reads to minimize mismatched bases using RealignerTargetCreator and realigned indels (insertions and deletions) using IndelRealigner in the Genome Analysis Toolkit (GATK; McKenna et al. 2010). We identified single nucleotide polymorphisms (SNPs) and indels using the GATK UnifiedGenotyper, annotated SNPs with VariantAnnotator, and masked indels using VariantFiltration. We removed SNPs with a quality score below Q30 and conducted read-backed phasing using GATK. We outputted SNPs in vcf format and used the program `add_phased_snps_to_seqs_filter.py` from `seqcap_pop` to insert SNPs into reference sequences and to produce alignments for each locus across individuals. SNPs on the same locus for which phasing failed were inserted using the appropriate International Union of Pure and Applied Chemistry (IUPAC) ambiguity codes. We collated sequences and produced final alignments using Multiple Alignment using Fast Fourier Transform (MAFFT; Katoh et al. 2005). Python scripts (available at [https://github.com/mgharvey/seqcap\\_pop](https://github.com/mgharvey/seqcap_pop)) were used to make input files for G-PhoCS (Gronau et al. 2011) and Structure (Pritchard et al. 2000).

### Quantifying Genetic Differentiation and Population Structure

With the UCE data, we used a discriminant analysis of principal components (DAPC) to identify clusters of genetically related individuals (Jombart et al. 2010). This is a computationally fast, multivariate method designed for large genomic datasets (Jombart et al. 2010). DAPC assigns individuals to genetic groups by maximizing the differences between groups and minimizing the variation within a group (Jombart et al. 2010).

In addition to DAPC, we estimated the number of populations and conducted population assignment of individuals using all UCE SNPs and the linkage model in Structure 2.3.4 (Pritchard et al. 2000). Structure assigns individuals into a user-defined number of populations ( $K$ ) and gives likelihood values for each data partition to those populations under an assumption of Hardy-Weinberg equilibrium. We did not assign individuals to populations

a priori. After an initial burn-in of 10,000 generations, we used 500,000 Markov chain Monte Carlo (MCMC) generations in the Structure analyses. We performed 10 replicates each for  $K = 1-5$ . Structure Harvester (Earl and vonHoldt 2012) was used to summarize the Structure output, to implement the Evanno method (Evanno et al. 2005), and to produce input files for CLUMPP (Jakobsson and Rosenberg 2007). Structure uses a stochastic algorithm, which can result in individuals being assigned to different populations across replicates (Jakobsson and Rosenberg 2007). Using the CLUMPP *FullSearch* algorithm, we found the optimal alignment across the Structure analyses. We used R (R Core Team 2015) and the CLUMPP output file with sample assignments to visually display results. To assess whether finer levels of population structure were present within *inornata* and *semipalmata*, we ran additional Structure analyses (again with  $K = 1-5$ ) on datasets composed of just *inornata* or just *semipalmata* individuals.

### Summary Statistics

We estimated standard population genetic summary statistics from UCE alignments using the Bio.PopGen module in Biopython (Cock et al. 2009). We used the 14,285 SNPs with  $\geq 50\%$  complete data across individuals (74% of the total number of SNPs) to identify SNPs with alleles that were fixed between the 2 subspecies and to calculate the fixation index ( $F_{ST}$ ) for each locus.  $F_{ST}$  is a measure of the within-population genetic variance relative to the total genetic variance, where  $F_{ST} = 1 - (\text{mean distance between sequences within populations} / \text{mean distance between sequences between populations})$ .  $F_{ST}$  values range from 0 to 1; a value of 0 indicates that populations are interbreeding freely, and an  $F_{ST}$  of 1 suggests that populations do not share any alleles.

Fixed differences between the subspecies might have been present in our sample simply due to the limited sample size. To test the proportion of the fixed differences that could have been explained just by sampling, we examined the number of fixed differences that were present when each of the individuals were randomly assigned to populations of the same size as the actual sampled populations. We conducted this permutation test 1,000 times and compared the number of fixed differences with random assignments to the number observed with the actual population assignments.

We conducted coalescent simulations to assess how the observed number of fixed differences at UCE loci compared with the expected number of fixed differences given the level of mitochondrial divergence and the differences in effective population size between the mitochondrion, Z-linked, and autosomal markers. To build the expected distribution we simulated 1,000 datasets of the same size as the empirical dataset using the divergence

time estimated from the mitochondrial data and the demographic history estimated from the UCE alignments and counted the number of fixed SNPs in each replicate. We simulated the mapping of loci to the Z chromosome using effective population sizes that were  $\frac{3}{4}$  of the size of autosomal loci, and the number of simulated samples from these loci reflected the reduced number of chromosomes due to heterogamy of females in the empirical dataset.

### Estimation of Demographic History

To estimate current and historical population sizes and migration rates, we used program G-PhoCS 1.2.1 (Gronau et al. 2011), which is a Bayesian MCMC program for large datasets. We examined models with no migration between populations subsequent to divergence and a model allowing for migration. We used all individuals in G-PhoCS analyses and randomly selected 1,000 loci ( $\sim 22\%$  of the total) to increase the number of iterations and to reduce computation time. We used 2,000,000 iterations, removed 10% of the iterations as burn-in, and evaluated convergence in Tracer (Rambaut et al. 2014) to ensure that all effective sample size (ESS) values were greater than 200. Following the supplementary material in Gronau et al. (2011), effective population size ( $N_e$ ) was calculated with  $\theta = 4N_e\mu$ , where  $\mu$  is mutation rate per nucleotide site per generation. Migration rates were calculated with the migration rate per generation parameter ( $m_{sx} \times \theta_x/4 = M_{sx}$ ), which is the proportion of individuals in population  $x$  that arrived by migration from population  $s$  per generation. Standardized substitution rates are not available for UCE loci, so we determined the substitution rate by setting the UCE divergence time to the divergence time inferred from the dating analysis of mitochondrial data (see below). This substitution rate was used for all parameter calibrations.

### Assessing the Genomic Distribution of Fixed Alleles

To assess the genomic context of genetic differences between Willet subspecies, we mapped the UCE loci to the closest available genome assembly, that of the Killdeer (*Charadrius vociferus*; Gilbert et al. 2014). Because the Killdeer genome is in 15,167 scaffolds (fragments of unknown order and relative position) rather than assembled into chromosomes, we also mapped the loci to the phylogenetically closest available genome that has chromosome assemblies (Zebra Finch [*Taeniopygia guttata*]; Warren et al. 2010). We mapped loci using Blastn (Altschul et al. 1990) with stringent similarity settings, and we conducted analyses based on the mapping position with the highest bit score for each locus. We were particularly interested in the relative frequency of fixed alleles on sex-linked loci mapping to the Z chromosome relative to the autosomal loci. Given some number of fixed SNPs between populations, a subset are expected to occur on UCEs mapping to the Z chromosome due to chance. To test

whether this could explain the observed distribution of fixed alleles, we permuted the locations of fixed SNPs across the positions of all recovered UCE loci 1,000 times. We corrected for the  $\frac{3}{4}$  effective population size of the avian Z chromosome relative to the autosomes and for the fact that we had  $\frac{3}{4}$  the number of chromosomes sampled at the Z chromosome loci relative to autosomal loci because some of the sampled individuals were females.

### Evaluating Support for Alternative Species Limits

We conducted Bayes factor species delimitation using SNPs with the method BFD\* (Grummer et al. 2014, Leaché et al. 2014). BFD\* uses Bayes factors (Kass and Raftery 1995) to compare the marginal likelihoods of phylogenies in which sample assignments to species differ in order to evaluate support for different species delimitation strategies. We used the species tree method SNAPP (Bryant et al. 2012) included in BEAST 2 (Bouckaert et al. 2014) and path sampling to evaluate the marginal likelihoods of trees in which the 2 Willet subspecies were either treated as separate or combined into 1 taxon. To streamline computation, we selected only *inornata* samples from the breeding grounds ( $n = 6$ ) and the same number of breeding *semipalmata* samples for analysis. As an out-group, we used UCE sequences from the Killdeer genome, extracted with Blastn (Altschul et al. 1990) and aligned to Willet sequences using MAFFT (Katoh et al. 2005). We selected a single SNP from each UCE locus for analysis to maximize marker independence. Path sampling analyses were run for 12 and 24 steps with 100,000 MCMC generations following 10,000 pre-burn-in generations. Log-normal distributions were used for the prior on the parameter (lambda) governing species divergence rate and for the rate priors.

### Mitochondrial Data

We sequenced 1,041 base pairs (bp) of the mitochondrial gene NADH dehydrogenase subunit 2 (ND2; H05216; Hackett 1996; H06313; Sorenson et al. 1999) for 8 *inornata* and 10 *semipalmata* individuals. In 2 samples (LSUMZ 5985 and LSUMZ 71708), only the forward sequencing reaction was successful (see Table 1 for GenBank accession numbers). ND2 was amplified via the following polymerase chain reaction (PCR) protocol for 25  $\mu$ l reactions: denaturation stage at 94°C for 5 min, followed by 34 cycles of 94°C for 30 s, 60 s of annealing at 50°C and 60 s at 72°C, and termination with a 10 min 72°C elongation. PCR products were purified and Sanger sequenced at Beckman-Coulter (Danvers, Massachusetts, USA). Geneious 9.0.2 (<http://www.geneious.com>, Kearse et al. 2012) was used to evaluate and trim chromatograms, and MUSCLE (Edgar 2004) was used to align consensus sequences.

We used ND2 sequence data obtained from GenBank and the sequence data generated for this project to build a



phylogeny of the genus *Tringa* using the Spotted Sandpiper (*Actitis macularius*) as an outgroup (Pereira and Baker 2005, Gibson and Baker 2012; see Appendix Table 4 for the taxa used and GenBank accession numbers). To determine the best-fit finite-sites substitution model for the dataset, we used jModelTest 2.1.6 (Guindon and Gascuel 2003, Darriba et al. 2012). The jModelTest 2.1.6 likelihood settings included 3 substitution schemes, base frequencies, gamma rate variation, and base tree search. We used maximum likelihood phylogenetic analysis in RAXML (Stamatakis 2014) and the general time reversible model with gamma distributed rate variation (GTR +  $\Gamma$ ) substitution model, and we ran 1,000 bootstrap replicates to estimate a tree. We also used a Bayesian phylogenetic approach to estimate a time-calibrated tree in BEAST 2 (Bouckaert et al. 2014). We used a relaxed log-normal clock with a mean of 2.5% per million years (Smith and Klicka 2010) and the GTR +  $\Gamma$  substitution model for calibration. We ran the Bayesian analysis for 100 million iterations, sampling every thousand, checked convergence in Tracer (Rambaut et al. 2014), and estimated a maximum clade credibility tree after removing the first 10% of samples as burn-in. Finally, we built an ND2 haplotype network in PopART (Leigh and Bryant 2015) using the Templeton, Crandall, and Sing (TCS) algorithm (Clement et al. 2002).

### Variation in Morphological Characters

To quantify morphological differences between subspecies, we measured tarsus length, wing chord, exposed culmen, bill depth at the distal end of nares, and the ratio of bill depth at the distal end of nares to exposed culmen in 5 nonbreeding *inornata* from California, Florida, and Louisiana, 5 breeding *inornata* from Wyoming, and 7 *semipalmata* (6 breeding and 1 nonbreeding) from Louisiana. The individuals measured were represented in our genomic dataset, so we were able to corroborate subspecific identification based on morphometric and genomic data. To determine whether mensural characters clustered with the subspecific designations of the individuals in our dataset, we used a principal components analysis (PCA) in ggplot2 (Wickham 2009), with an ellipse probability of 0.95. We used a linear discriminant function analysis in the MASS package (Venables and Ripley 2002) in R to evaluate how well a priori groupings of individuals by subspecies were supported by measured morphological characters.

## RESULTS

### Population Genetic Differentiation and Structure

Of the 4,635 UCE loci that we recovered, 283 were invariant (see [Supplemental Material](#)). The variable loci contained 19,322 SNPs. From the total sample of 30

individuals, we identified 42 loci containing 43 SNPs that were fixed for alternate alleles between *semipalmata* and *inornata* (Appendix Table 5). This is a significantly higher number of fixed SNPs than would be expected simply due to our sample sizes ( $P < 0.001$ ). Assuming the divergence time between populations estimated from mtDNA and the demographic history estimated from the empirical UCE data using G-PhoCS, the number of fixed nuclear SNPs fell within the expected range. DAPC analyses recovered 2 distinct genetic clusters that separated in multivariate space and corresponded to the 2 subspecies based on the distribution- and morphology-based specimen identifications (Figure 2A). The optimal number of populations ( $K$ ) in the Structure analysis was 2, based on  $\Delta K$  and likelihood scores. The individual assignments revealed 2 distinct groups corresponding to the 2 subspecies, with no individuals of mixed ancestry (Figure 2B, Appendix Table 6). Structure analyses run separately on the *inornata* and *semipalmata* datasets recovered an optimal  $K$  of 1 for both subspecies (Appendix Table 7). Average per-locus  $F_{ST}$  was 0.14, but with wide variation across loci ( $SD = 0.31$ ;  $F_{ST}$  for each locus can be found in [Supplemental Material](#); for a histogram of  $F_{ST}$  values see Appendix Figure 6).

### Effective Population Size and Migration

When divergence time between Willet subspecies from G-PhoCS was normalized to the value from the time-calibrated Bayesian phylogenetic tree from ND2, the inferred UCE substitution rate was  $2.59 \times 10^{-4}$  substitutions per million years (95% highest posterior density =  $9.52 \times 10^{-5}$  to  $6.50 \times 10^{-4}$ ). G-PhoCS results indicated that the effective population size ( $N_e$ ) was on average 2.5 times greater for *inornata* than for *semipalmata* (Table 2). Both subspecies had a higher inferred  $N_e$  than their ancestral population (Table 2). Mean migration rates estimated using G-PhoCS were close to 0 (0.0002 individuals per generation; Table 2).

### ND2 Phylogeny and Network

In the time-calibrated BEAST 2 tree, the 2 Willet subspecies diverged 729,799 yr ago (95% highest posterior density = 290,328 to 1,411,779 yr ago; Figure 3A; see Appendix Figure 7 for expanded RAXML and BEAST phylogenies). Based on our limited sample size, 5 base-pair differences separated the mitochondrial haplotype groups of the 2 subspecies (Figure 3B), and *inornata* showed greater ND2 haplotype diversity (Appendix Table 8). The subspecies exhibited 0.85% mean sequence divergence (Appendix Table 9).

### UCE Mapping

Of the 4,635 UCE loci, 4,611 (99.5%) successfully mapped to the Killdeer genome and 4,607 (99.4%) to the Zebra Finch genome. We identified 42 UCE loci containing 43

**TABLE 2.** The demographic history of all *Tringa semipalmata* individuals ( $n = 30$ ) in our dataset based on 1,000 ultraconserved element (UCE) loci estimated using program G-PhoCS (Gronau et al. 2011). Mean effective population size ( $N_e$ ) is given for the eastern (*semipalmata*) and western (*inornata*) subspecies; ancestral  $N_e$  is also shown. Migration is migrants per generation. The 95% lower and higher highest probability density (HPD) values are the predictive distribution of estimates and are equivalent to confidence intervals.

	$N_e$ <i>semipalmata</i>	$N_e$ <i>inornata</i>	Ancestral $N_e$	Migration from <i>semipalmata</i> to <i>inornata</i>	Migration from <i>inornata</i> to <i>semipalmata</i>
Mean	790,616	2,004,047	694,411	$2.10 \times 10^{-4}$	$7.69 \times 10^{-5}$
95% HPD, lower	734,827	1,887,248	645,004	$9.76 \times 10^{-9}$	$1.90 \times 10^{-9}$
95% HPD, upper	846,598	2,127,420	745,366	$6.56 \times 10^{-4}$	$2.43 \times 10^{-4}$

SNPs fixed for alternate base pairs between *inornata* and *semipalmata* (Appendix Table 5). When mapped to the Zebra Finch genome assembly, 17 of the SNPs with fixed differences mapped to the Z chromosome (Appendix Table 5, Figure 4). Based on 1,000 permutations of the distribution of fixed SNPs across UCE loci, the number of loci with fixed SNPs on the Z chromosome was disproportionately high ( $P < 0.001$ ), even when we accounted for the higher probability of fixation due to the  $\frac{3}{4}$  effective population size of the Z chromosome relative to the autosomes and the reduced sample from the Z chromosome due to the female individuals in our dataset (Appendix Figure 8). Similarly, coalescent simulations indicated that the frequency of fixed SNPs on the Z chromosome was higher than would be explained by neutral processes ( $P = 0.002$ ).

Twenty of the 42 loci with fixed SNPs, including 4 of the 17 loci that occurred on the Z chromosome, were within predicted exons based on the annotations in the Killdeer genome (Gilbert et al. 2014). The number of fixed SNPs within exons was higher than expected by chance ( $P = 0.013$ ; Appendix Figure 9). However, the UCE alignments with fixed SNPs did not contain open reading frames, which complicated a more detailed assessment of the function of these loci or a determination of whether the substitutions were silent or not. It is also important to note that the apparent absence of open reading frames in these loci could have been due to errors in the assemblies that resulted in frame shifts, to differences between Willets and Killdeer in the locations of functional genes, to erroneous mapping results, or to errors in the exon predictions in the Killdeer genome.

**Species Delimitation**

Bayes factor species delimitation strongly supported the treatment of the 2 Willet subspecies as separate species (Bayes factor = 978, with Bayes factors  $> 10$  generally considered decisive; Kass and Raftery 1995). For each species delimitation scheme, the marginal likelihood values differed little between path sampling analyses involving 12 and 24 steps, suggesting that the runs of 24 steps were sufficient for accurate marginal likelihood estimation.

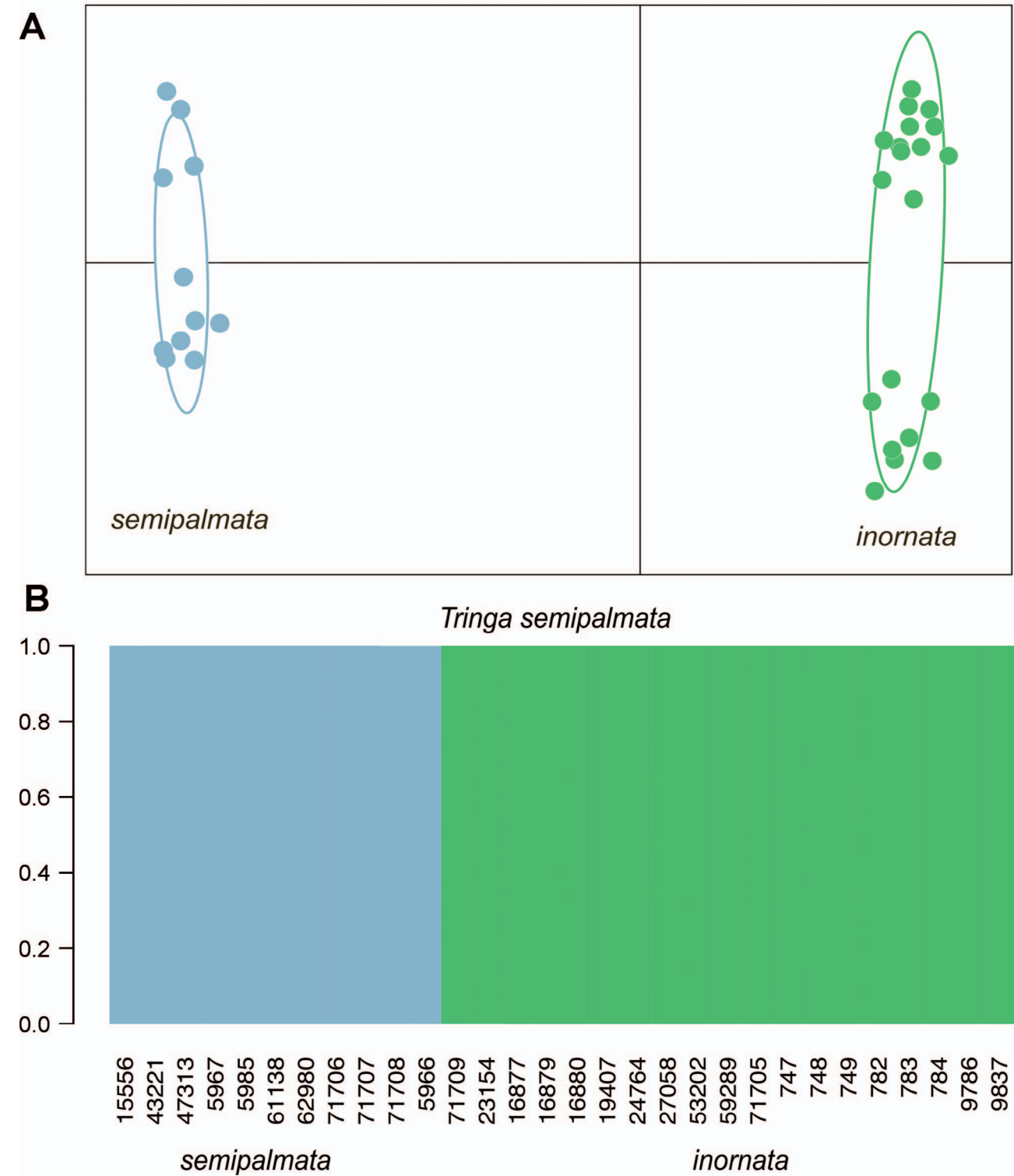
**Variation in Morphological Characters**

Mean measurements of morphological characters corresponded to subspecies ranges listed by Pyle (2008): *inornata* = wing chord  $>200$  mm, tarsus  $>60$ , exposed culmen 55–67 mm; *semipalmata* = wing chord  $<200$  mm, tarsus  $<60$  mm, exposed culmen 47–61 mm. Western (*inornata*) individuals had a longer wing chord (mean 210 mm vs. *semipalmata* = 199 mm) and longer exposed culmen (59 mm vs. *semipalmata* = 58 mm). Mean tarsus length also was longer in *inornata* (63 mm vs. 56 mm, respectively). The bill depth at the distal edge of the nares was slightly different between the 2 subspecies (*inornata* = 7.6 mm; *semipalmata* = 8.1 mm). The ratio of bill depth at the distal edge of nares to exposed culmen is a character used to diagnose the 2 subspecies ( $>0.18$  mm in *semipalmata*,  $<0.18$  in *inornata*; Pyle 2008), but we found little difference in the ratio between subspecies: *semipalmata* = 0.14 mm and *inornata* = 0.13 mm (Table 3). PC1 explained 80% of the variation in the dataset, and PC2 explained 13% of the variation in the dataset (Figure 5, Appendix Table 10). Both principal components were composed of tarsus length, wing chord, and exposed culmen, with different loading values. Based on linear discriminant analysis, the measured morphological characters predicted subspecies designations 94% of the time (*inornata* = 100%, *semipalmata* = 86%).

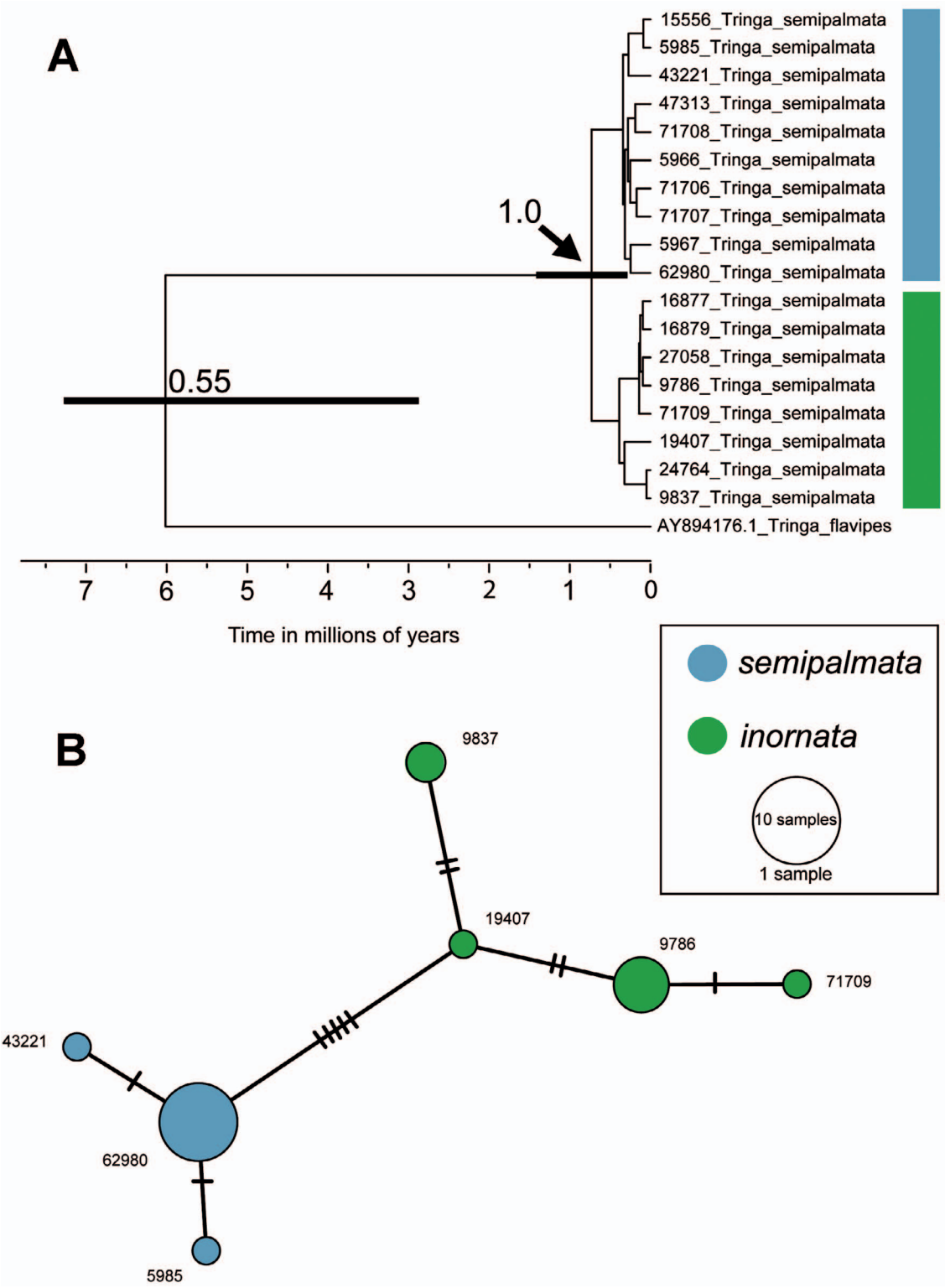
**DISCUSSION**

The geographically closest breeding populations of the 2 Willet subspecies (central Texas Coast and northwest Colorado) are separated by  $>1,600$  km. We found no evidence of hybridization or introgression between them. Individuals of each subspecies clustered together based on morphological, mitochondrial, and nuclear data, and there was no evidence of hybridization or introgression between the 2 subspecies. They displayed a relatively shallow 0.85% mitochondrial sequence divergence, which supported a divergence time of at least  $\sim 300,000$  yr ago, and had 42 nuclear loci (of 4,635) with fixed differences based on our sampling. Both subspecies have experienced an increased effective population size ( $N_e$ ) through time. The 2.5 times

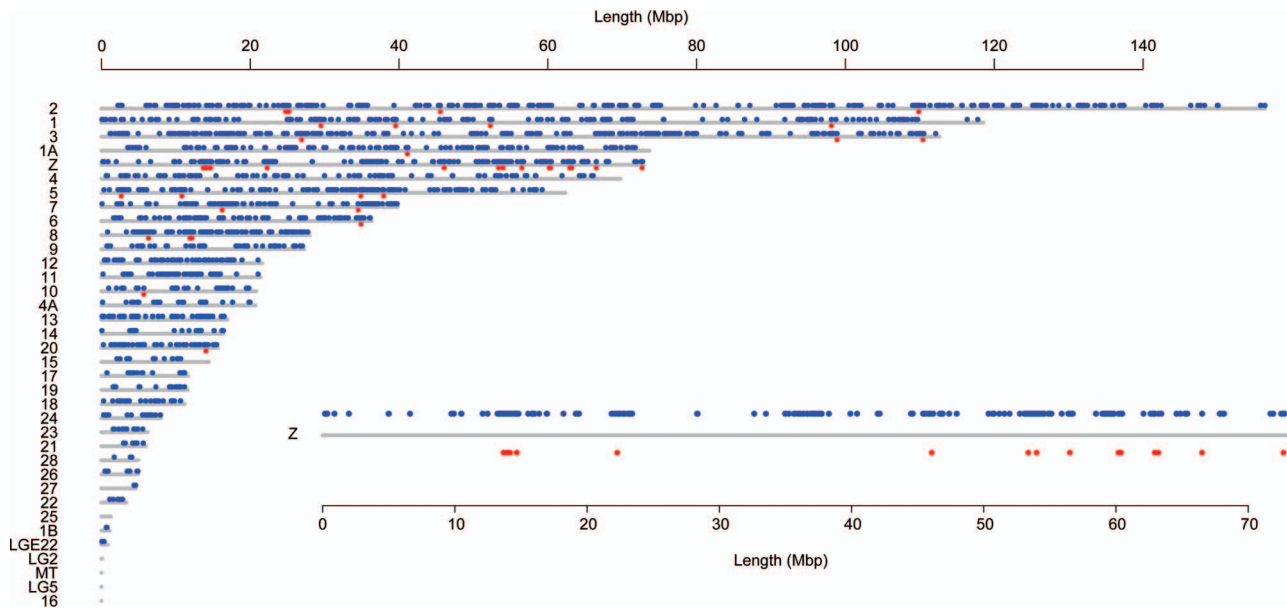




**FIGURE 2.** Results of (A) discriminant analysis of principal components (DAPC) and (B) program Structure analysis of genetic differentiation between *Tringa semipalmata* subspecies *inornata* and *semipalmata* sampled in Wyoming, California, Florida (*inornata*), New York, Texas (*semipalmata*), and Louisiana, USA (both subspecies). *Tringa semipalmata semipalmata* (the eastern subspecies) individuals are in blue, and *T. s. inornata* (the western subspecies) individuals are in green.



**FIGURE 3.** (A) The ND2 BEAST2 phylogeny of *Tringa semipalmata semipalmata* and *T. s. inornata* (sampled in Wyoming, California, Florida [*inornata*], New York, Texas [*semipalmata*], and Louisiana [both subspecies]) and their sister species, *Tringa flavipes*. Posterior probabilities are indicated at the nodes. Phylogenies with broader taxonomic sampling can be found in Appendix Figure 7. (B) ND2 haplotype network for *Tringa semipalmata*. Individuals with identical sequences to the individual listed on the haplotype network can be found in Appendix Table 8.



**FIGURE 4.** Ultraconserved elements (UCEs; blue dots) of *Tringa semipalmata semipalmata* and *T. s. inornata* sampled in Wyoming, California, Florida (*inornata*), New York, Texas (*semipalmata*), and Louisiana (both subspecies) plotted across the Zebra Finch (*Taeniopygia guttata*) genome. Chromosome length is represented in megabase pairs (Mbp). The red dots represent the positions of UCEs with fixed alleles. Chromosome names are on the y-axis.

higher  $N_e$  and higher haplotype diversity in the *inornata* subspecies likely reflects the larger breeding range of *inornata*. Collectively, our data indicate that the 2 Willet subspecies are on separate evolutionary trajectories and have marked ecological, behavioral, morphological, and genetic differences, consistent with their treatment as distinct evolutionary units.

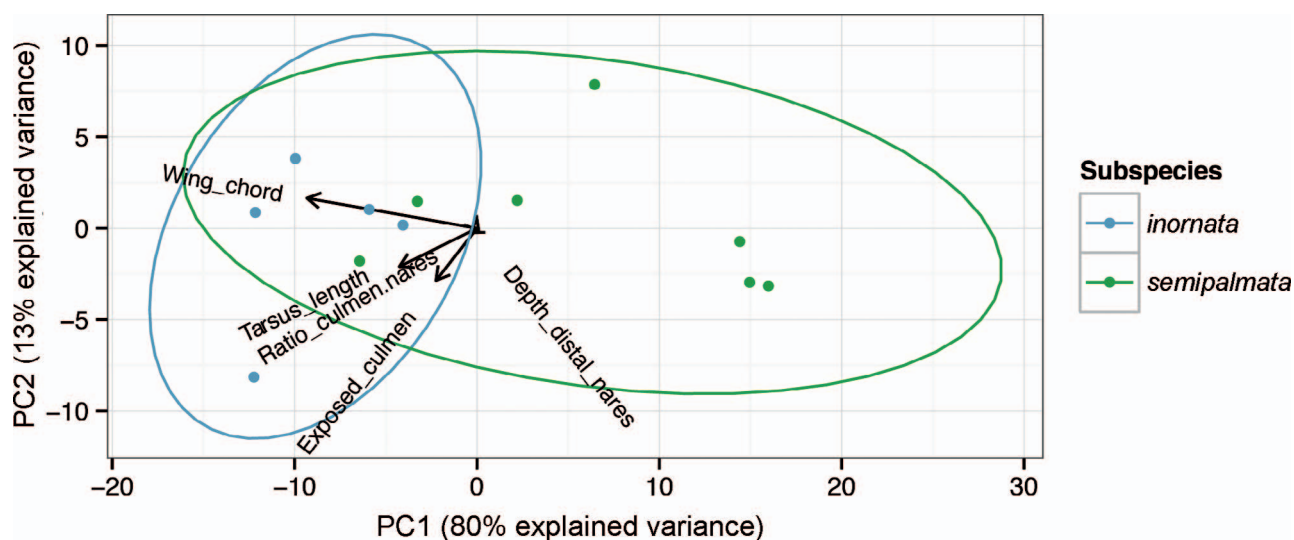
**Genomic Data and Species Delimitation**

The 0.85% mitochondrial sequence divergence between the Willet subspecies is relatively shallow and does not meet the often-implemented divergence threshold of >2% for species-level splits (Johns and Avise 1998, Hebert et al. 2004). However, Bayesian species delimitation based on UCEs indicates strong support for the treatment of the

**TABLE 3.** Morphological measurements for specimens of *Tringa semipalmata* in our genomic dataset housed at the Louisiana State University Museum of Natural Science (LSUMZ) and University of Wyoming Museum of Vertebrates (UWYMV). Measurements are in mm. See Table 1 for collection locality and breeding condition information.

Museum	<i>Tringa semipalmata</i> subspecies	Skin catalog number	Tissue number	Sex	Tarsus length	Wing chord	Exposed culmen	Depth at distal nares	Ratio of bill depth at distal nares to exposed culmen
LSUMZ	<i>inornata</i>	156047	16877	Male	62.9	210	58.4	7.5	0.13
LSUMZ	<i>inornata</i>	156049	16879	Male	62.0	215	58.1	6.9	0.12
LSUMZ	<i>inornata</i>	159110	19407	Female	63.2	216	61.7	7.1	0.12
LSUMZ	<i>inornata</i>	185324	71705	Female	62.6	208	58.6	8.1	0.14
LSUMZ	<i>inornata</i>	185328	71709	Female	69.3	212	67.3	8.0	0.12
UWYMV	<i>inornata</i>	2808	747	Unknown	61.9	208	58.2	7.5	0.13
UWYMV	<i>inornata</i>	2809	748	Female	61.2	207	61.0	7.1	0.12
UWYMV	<i>inornata</i>	2810	749	Female	62.0	211	60.1	8.2	0.12
UWYMV	<i>inornata</i>	2812	782	Female	63.7	206	50.2	8.0	0.16
UWYMV	<i>inornata</i>	2811	784	Male	62.1	211	57.9	7.2	0.13
LSUMZ	<i>semipalmata</i>	151993	15556	Male	55.3	189	57.5	7.7	0.13
LSUMZ	<i>semipalmata</i>	173598	43221	Male	52.4	192	58.0	8.3	0.14
LSUMZ	<i>semipalmata</i>	175755	47313	Male	55.7	190	57.6	7.7	0.13
LSUMZ	<i>semipalmata</i>	183976	61138	Male	53.9	202	51.0	7.7	0.15
LSUMZ	<i>semipalmata</i>	185325	71706	Female	57.0	209	61.5	9.0	0.15
LSUMZ	<i>semipalmata</i>	185326	71707	Male	55.3	204	59.8	8.1	0.14
LSUMZ	<i>semipalmata</i>	185327	71708	Female	61.7	210	63.1	8.4	0.14





**FIGURE 5.** Principal components analysis (PCA) of morphological characters of *Tringa semipalmata semipalmata* and *T. s. inornata* sampled in Wyoming (*inornata*), California (*inornata*), Texas (*semipalmata*), and Louisiana (both subspecies). Ellipses are 95% confidence intervals. The rotated component matrix and the standard deviation and proportion of variance explained by the components can be found in Appendix Table 10.

Willet subspecies as separate species. Many studies indicate that taxa cannot be diagnosed based solely on genetic divergence using standard molecular clocks from a few loci (see Lovette 2004), and this is becoming increasingly clear through studies that use genome-scale datasets (e.g., Ellegren et al. 2012, Dhimi et al. 2016). The differences in divergence between autosomal and Z-linked loci demonstrate variation in divergence across the Willet genome that would seem to preclude using a single sequence difference threshold applied to one or a few loci for species delimitation.

### Z Chromosome Divergence

High divergence on the Z chromosome between taxa is becoming a widespread observation in studies of genome-wide divergence between bird species (Ellegren et al. 2012, Lavretsky et al. 2015, Dhimi et al. 2016). However, the source of this pattern is still unclear. In birds, the male is the homogametic sex (ZZ), whereas females are heterogametic (ZW). In monogamous species with similar sex ratios, the  $N_e$  of the Z chromosome is  $\frac{3}{4}$  that of autosomes (Charlesworth et al. 1987), which results in faster fixation of new mutations and may lead to higher divergence. Even when accounting for smaller  $N_e$ , we observed high rates of Z chromosome divergence between Willet subspecies relative to expected rates under purely neutral processes. Reproductive skew in males of species with sexual selection further reduces the  $N_e$  of the Z chromosome and produces a faster Z effect (Charlesworth 2009, Ellegren 2009, Wright and Mank 2013), which may explain the observed Z chromosome divergence in Willets.

are thought to be monogamous (Howe 1982), although males engage in group displays and female behavior has been interpreted as advertisement for extrapair copulation (Howe 1982, Douglas 1996, Lowther et al. 2001), both of which could promote extrapair copulations and thus variance in male mating success that may result in faster Z evolution (Wright et al. 2015).

Adaptive evolution also could be responsible for high rates of Z chromosome evolution between populations, if divergent selection is greater at Z-linked loci. Alleles at Z-linked loci are unmasked in hemizygous females; therefore, recessive beneficial alleles may be rapidly fixed by positive selection relative to alleles at autosomal loci. Differences in morphology, plumage, and behavior between the Willet subspecies combined with their different habitats and ecologies may be evidence of the potential for evolution in response to natural selection in these populations. If sexual selection is occurring and sexually selected characteristics are Z-linked, then adaptive evolution may occur at even higher rates in the Z chromosome (Sæther et al. 2007, Pryke 2010, Schroeder et al. 2010, Toms et al. 2012, Dhimi et al. 2016). Male and female Willets look similar, but females are larger on average and the sexes differ in voice and behavior during the breeding season (Lowther et al. 2001). Overall, teasing apart the various potential causes of higher rates of divergence on sex-linked chromosomes is challenging (Meisel and Connallon 2013), and additional data will be required to investigate the potential for adaptive, sex-linked divergence between Willet subspecies.

## Concluding Remarks

In addition to the already recognized differences in ecology, morphology, and song, our data show genomic divergence between *inornata* and *semipalmata*. We found no evidence of ongoing hybridization that would lead to detectable levels of genetic introgression. Additional research should explore the possibility that genetic structure exists within 1 or both of the 2 Willet subspecies. Our study lacked samples from Caribbean populations and populations in many parts of the breeding ranges of both subspecies. In addition, *inornata* populations show fidelity to breeding and wintering grounds, which could lead to geographic isolation and genetic structure. The *inornata* that breed in the Great Basin wetlands show philopatry to breeding grounds and to wintering grounds in coastal and estuarine habitats in northern California (Haig et al 2002), and the *inornata* that breed in Alberta winter in Mexico and Costa Rica (Lowther et al. 2001, Haig et al. 2002). It is unknown whether *semipalmata* also exhibit philopatry. With regard to species status, reciprocal playback studies in populations of both subspecies would be illuminating.

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**Ethics statement:** This research was conducted in compliance with all required research and collecting permits.

**Author contributions:** J.A.O. wrote the paper, collected and analyzed data, and assisted in the design of the project; M.G.H. developed methods, assisted in data analyses and design, and assisted in writing the manuscript; R.C.R. and D.U.F. assisted with data collection in the lab; D.L.D. and S.W.C. assisted in project design and collected specimens;

L.C.M. contributed tissues and morphological data; M.D.C. contributed tissues and morphological data; R.T.B. conceived of the project, assisted in the design, contributed substantial materials and resources, and assisted in writing the manuscript.

**Data statement:** UCE raw read data are available on NCBI SRA SRP071703 (BioProject PRJNA314305). UCE sequence alignments are available on Dryad (<http://dx.doi.org/10.5061/dryad.2361d>)

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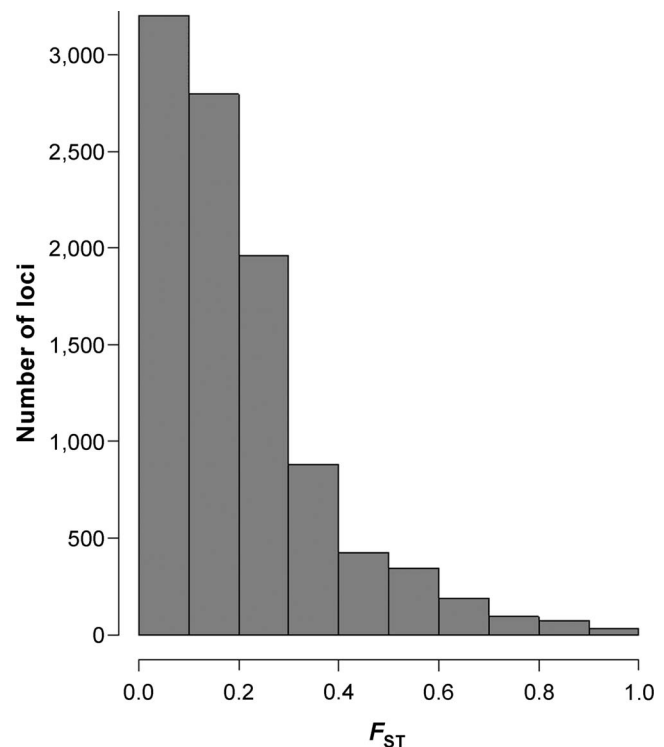
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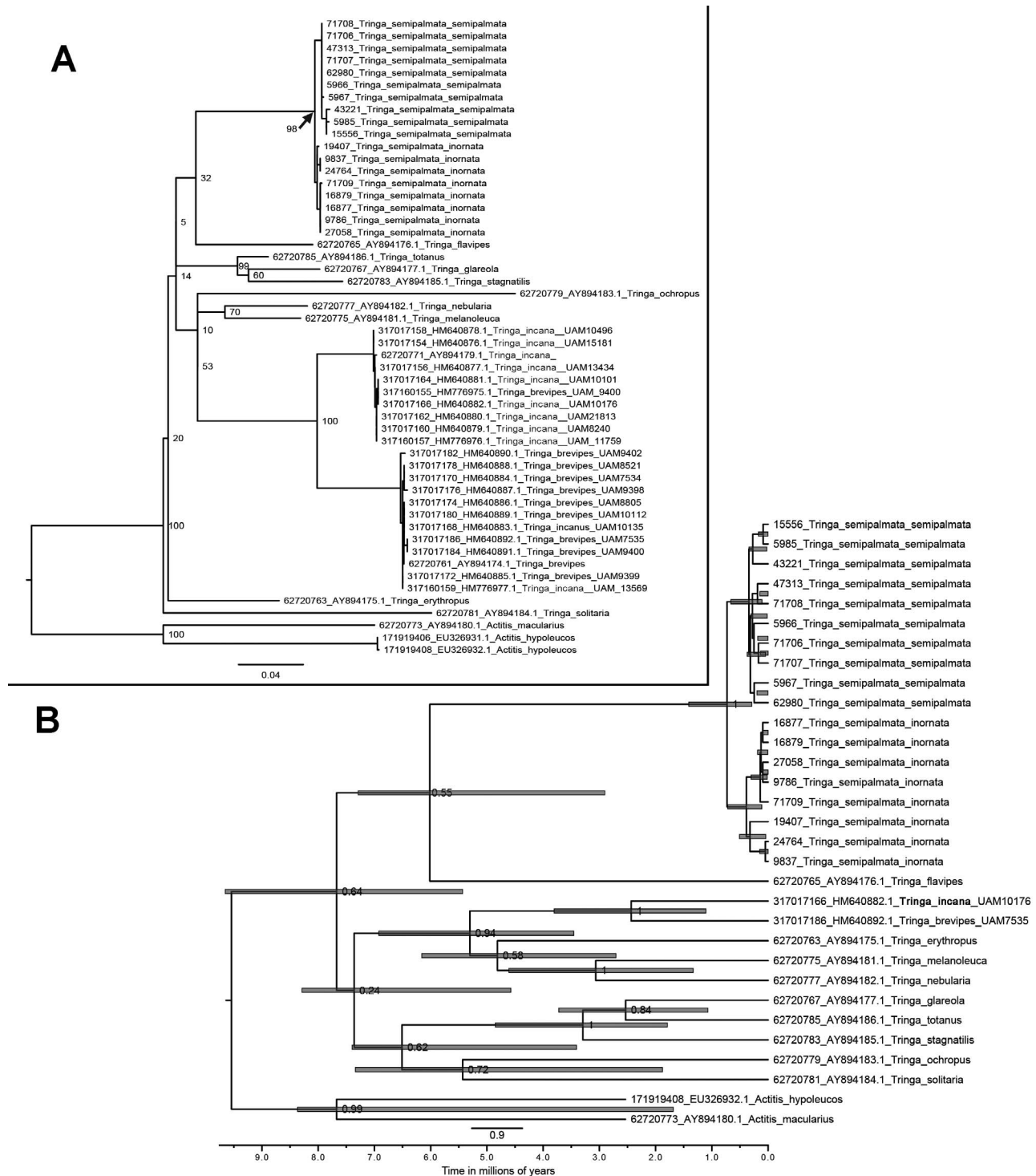


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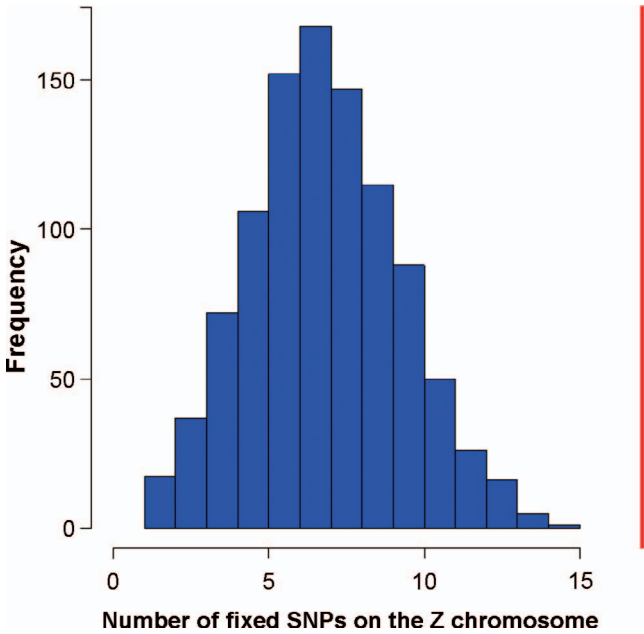


**APPENDIX FIGURE 6.** Histogram of the 4,000 ultraconserved element (UCE) loci with nonnegative  $F_{ST}$  values in our dataset for *Tringa semipalmata semipalmata* and *T. s. inornata* sampled in Wyoming, California, Florida (*inornata*), New York, Texas (*semipalmata*), and Louisiana (both subspecies).

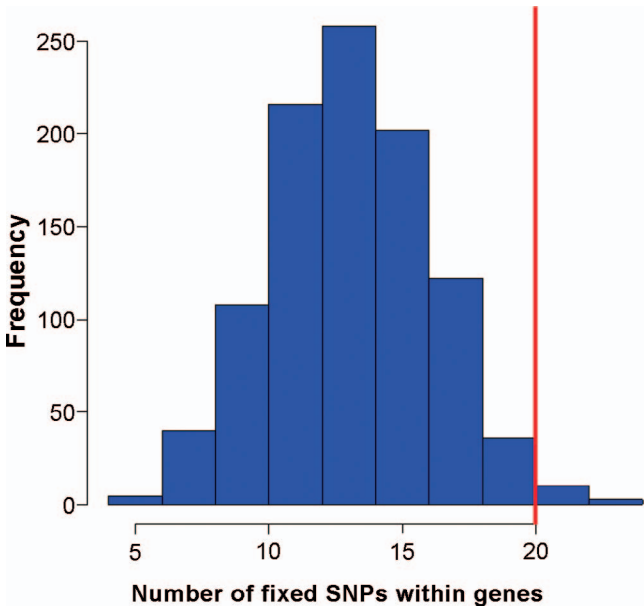


**APPENDIX FIGURE 7.** Expanded ND2 phylogenies of *Tringa semipalmata semipalmata* and *T. s. inornata* sampled in Wyoming, California, Florida (*inornata*), New York, Texas (*semipalmata*), and Louisiana (both subspecies) and all other members of the genus *Tringa* available on GenBank obtained with (A) program RAXML (Stamatakis 2014) and (B) program BEAST 2 (Bouckaert et al. 2014). The trees are rooted with *Actitis* species following Pereira and Baker (2005). In (A) node support is based on 1,000 bootstrap replicates, and in (B) posterior probabilities are shown on the nodes.





**APPENDIX FIGURE 8.** Simulation (blue bars) and observed (red line) numbers of fixed single nucleotide polymorphisms (SNPs) on the Z chromosome of ultraconserved elements (UCEs) from *Tringa semipalmata semipalmata* and *T. s. inornata* sampled in Wyoming, California, Florida (*inornata*), New York, Texas (*semipalmata*), and Louisiana (both subspecies).



**APPENDIX FIGURE 9.** Simulation (blue bars) and observed (red line) numbers of single nucleotide polymorphisms (SNPs) within genes of ultraconserved elements (UCEs) from *Tringa semipalmata semipalmata* and *T. s. inornata* sampled in Wyoming, California, Florida (*inornata*), New York, Texas (*semipalmata*), and Louisiana (both subspecies).

**APPENDIX TABLE 4.** GenBank (GenInfo Identifier [GI]) numbers and accession version from published sequence data included in phylogenetic analyses focused on *Tringa semipalmata semipalmata* and *T. s. inornata* sampled in Wyoming, California, Florida (*inornata*), New York, Texas (*semipalmata*), and Louisiana (both subspecies) and sister taxa within the genus *Tringa*. *Actitis* was used as an outgroup following Pereira and Baker (2005). Species are ordered by GI number.

GI	Accession version	Genus	Species	Voucher no.
171919406	EU326931.1	<i>Actitis</i>	<i>hypoleucos</i>	
171919408	EU326932.1	<i>Actitis</i>	<i>hypoleucos</i>	
317017154	HM640876.1	<i>Tringa</i>	<i>incana</i>	UAM15181
317017156	HM640877.1	<i>Tringa</i>	<i>incana</i>	UAM13434
317017158	HM640878.1	<i>Tringa</i>	<i>incana</i>	UAM10496
317017160	HM640879.1	<i>Tringa</i>	<i>incana</i>	UAM8240
317017162	HM640880.1	<i>Tringa</i>	<i>incana</i>	UAM21813
317017164	HM640881.1	<i>Tringa</i>	<i>incana</i>	UAM10101
317017166	HM640882.1	<i>Tringa</i>	<i>incana</i>	UAM10176
317017168	HM640883.1	<i>Tringa</i>	<i>incana</i>	UAM10135
317017170	HM640884.1	<i>Tringa</i>	<i>brevipes</i>	UAM7534
317017172	HM640885.1	<i>Tringa</i>	<i>brevipes</i>	UAM9399
317017174	HM640886.1	<i>Tringa</i>	<i>brevipes</i>	UAM8805
317017176	HM640887.1	<i>Tringa</i>	<i>brevipes</i>	UAM9398
317017178	HM640888.1	<i>Tringa</i>	<i>brevipes</i>	UAM8521
317017180	HM640889.1	<i>Tringa</i>	<i>brevipes</i>	UAM10112
317017182	HM640890.1	<i>Tringa</i>	<i>brevipes</i>	UAM9402
317017184	HM640891.1	<i>Tringa</i>	<i>brevipes</i>	UAM9400
317017186	HM640892.1	<i>Tringa</i>	<i>brevipes</i>	UAM7535
317160155	HM776975.1	<i>Tringa</i>	<i>brevipes</i>	UAM9400
317160157	HM776976.1	<i>Tringa</i>	<i>incana</i>	UAM11759
317160159	HM776977.1	<i>Tringa</i>	<i>incana</i>	UAM13569
62720761	AY894174.1	<i>Tringa</i>	<i>brevipes</i>	
62720763	AY894175.1	<i>Tringa</i>	<i>erythropus</i>	
62720765	AY894176.1	<i>Tringa</i>	<i>flavipes</i>	
62720767	AY894177.1	<i>Tringa</i>	<i>glareola</i>	
62720771	AY894179.1	<i>Tringa</i>	<i>incana</i>	
62720773	AY894180.1	<i>Actitis</i>	<i>macularius</i>	
62720775	AY894181.1	<i>Tringa</i>	<i>melanoleuca</i>	
62720777	AY894182.1	<i>Tringa</i>	<i>nebularia</i>	
62720779	AY894183.1	<i>Tringa</i>	<i>ochropus</i>	
62720781	AY894184.1	<i>Tringa</i>	<i>solitaria</i>	
62720783	AY894185.1	<i>Tringa</i>	<i>stagnatilis</i>	
62720785	AY894186.1	<i>Tringa</i>	<i>totanus</i>	

**APPENDIX TABLE 5.** Ultraconserved element (UCE) position of the 43 fixed single nucleotide polymorphisms (SNPs) of UCEs from *Tringa semipalmata semipalmata* and *T. s. inornata* sampled in Wyoming, California, Florida (*inornata*), New York, Texas (*semipalmata*), and Louisiana (both subspecies) on Zebra Finch (*Taeniopygia guttata*) chromosomes and Killdeer scaffolds. A scaffold is a series of contigs (contiguous sequences of DNA created by assembling overlapping sequenced fragments of a chromosome) and gaps that are in order but not necessarily connected in one continuous stretch of DNA sequence.

Locus	Position of fixed SNP	Killdeer			Zebra Finch		
		Scaffold	Start	End	Chromosome	Start	End
UCE 1497	70	NW_009648332.1	13298149	13298591	8	12173238	12172794
UCE 1631	472	NW_009648876.1	5870120	5870908	5	34871227	34872009
UCE 1787	627	NW_009648822.1	127284	127977	Z	13991263	13990589
UCE 1867	635	NW_009648876.1	5865681	5866382	5	34866848	34867570
UCE 1893	69	NW_009648822.1	386277	387021	Z	13744711	13744181
UCE 204	483	NW_009648250.1	4565871	4565319	Z	66511997	66512415
UCE 2051	70	NW_009661372.1	3210017	3210695	Z	22281436	22280824
UCE 2274	539	NW_009648712.1	3517479	3516724	2	109848860	109848088
UCE 2350	130	NW_009646761.1	2811102	2810340	1	39545671	39546386
UCE 2439	94	NW_009648252.1	878242	878534	Z	14684681	14684364
UCE 2845	453	NW_009649167.1	1794457	1793655	1	52271517	52270706
UCE 3104	671	NW_009648550.1	3938161	3937426	3	26901769	26902512
UCE 3181	505	NW_009646851.1	1967510	1966948	Z	46067413	46066982
UCE 3303	247	NW_009649706.1	1018126	1017522	Z	63206111	63206721
UCE 3314	177	NW_009661368.1	1665502	1664995	20	14057679	14057994
UCE 3381	70	NW_009648332.1	13582630	13582034	8	11900059	11900614
UCE 3471	350	NW_009648440.1	1974427	1974966	2	45549917	45550446
UCE 4098	527	NW_009648573.1	9021	9613	2	24764958	24764364
UCE 4143	670	NW_009648526.1	4519092	4519867	10	5701081	5700335
UCE 4733	103	NW_009648822.1	464976	465501	Z	13684125	13683641
UCE 4912	70	NW_009649040.1	484740	485414	2	25212227	25212812
UCE 5185	600	NW_009650176.1	2222294	2223078	6	34889906	34889323
UCE 5361	256	NW_009648250.1	2601515	2602215	Z	56512247	56512949
UCE 5456	524	NW_009647830.1	6430281	6429690	1A	41125292	41125886
UCE 5510	53	NW_009649572.1	58285	57741	Z	72670911	72670331
UCE 5576	535	NW_009648332.1	19776264	19776854	8	6333620	6333018
UCE 5821	538	NW_009660184.1	1510248	1509479	3	98873781	98873027
UCE 5829	64	NW_009649614.1	914873	914635	Z	53372952	53372433
UCE 5910	469	NW_009649951.1	2201075	2201659	Z	60225046	60224468
UCE 5956	404	NW_009650173.1	3762957	3763443	5	2669299	2668829
UCE 5956	419	NW_009650173.1	3762957	3763443	5	2669299	2668829
UCE 6242	142	NW_009648876.1	9205163	9204322	5	37935757	37935061
UCE 6390	309	NW_009649951.1	2037676	2038317	Z	60381755	60381100
UCE 6574	515	NW_009646428.1	246989	247568	Z	54001327	54000748
UCE 6800	45	NW_009646928.1	1838979	1838365	7	16238340	16238932
UCE 6826	436	NW_009650072.1	4223083	4223813	5	10817538	10816950
UCE 6915	581	NW_009649951.1	2246292	2245682	Z	60189699	60190278
UCE 694	139	NW_009648252.1	1418184	1417677	Z	14179514	14180013
UCE 6988	129	NW_009650060.1	2668146	2668871	7	34517856	34517241
UCE 7363	74	NW_009646936.1	1740170	1740705	1	98112113	98111666
UCE 7678	650	NW_009649193.1	214560	213838	3	110412021	110411197
UCE 7981	146	NW_009649706.1	1301179	1301933	Z	62937216	62936501
UCE 7985	242	NW_009647874.1	2898318	2897445	1	29511791	29511027

**APPENDIX TABLE 6.** Number of populations ( $K$ ), number of replicates, mean log-likelihood scores, log-likelihood standard deviation (SD), first- and second-order likelihood rate of change, and  $\Delta K$  values for  $K = 1$ –5 as determined by program Structure (Pritchard et al. 2000) for the complete *Tringa semipalmata* dataset.

$K$	Replicates	Mean $\text{Ln}P(K)$	SD $\text{Ln}P(K)$	$\text{Ln}'(K)$	$ \text{Ln}''(K) $	$\Delta K$
1	10	–320,962.2	6,068.3	NA	NA	NA
2	10	–264,330.2	16.7	56,632.0	76,298.1	4,582.5
3	10	–283,996.3	5,524.8	–19,666.1	15,147.6	2.7
4	10	–288,514.8	43,364.9	–4,518.6	99,083.2	2.3
5	10	–392,116.6	269,997.8	–103,601.7	NA	NA

**APPENDIX TABLE 7.** Number of populations ( $K$ ), number of replicates, mean log-likelihood scores, log-likelihood standard deviation (SD), first- and second-order likelihood rate of change, and  $\Delta K$  values for  $K = 1$ –5 as determined by program Structure (Pritchard et al. 2000) for the (A) *Tringa semipalmata inornata* and (B) *T. s. semipalmata* datasets.

$K$	Replicates	Mean $\text{Ln}P(K)$	SD $\text{Ln}P(K)$	$\text{Ln}'(K)$	$ \text{Ln}''(K) $	$\Delta K$
<b>(A) <i>inornata</i></b>						
1	10	–185,721.9	30.3	NA	NA	NA
2	10	–548,888.8	606,091.4	–363,166.9	603,425.9	1.0
3	10	–308,629.8	55,330.7	240,259.0	289,277.7	5.2
4	10	–357,648.4	123,007.9	–49,018.7	166,622.1	1.4
5	10	–573,289.2	283,514.4	–215,640.8	NA	NA
<b>(B) <i>semipalmata</i></b>						
1	10	–80,260.6	20.4	NA	NA	NA
2	10	–83,852.7	2,451.2	–3,592.1	8,095.1	3.3
3	10	–95,539.9	14,835.0	–11,687.2	28,158.4	1.9
4	10	–135,385.5	43,586.0	–39,845.6	134,611.3	3.1
5	10	–309,842.4	141,827.7	–174,456.9	NA	NA



**APPENDIX TABLE 8.** Identical ND2 sequences represented by node size in the haplotype network of *Tringa semipalmata*. The U.S. state in which the individual was collected follows the subspecies name: LA = Louisiana, NY = New York, TX = Texas, and CA = California.

Node label	Matching sequences
62980 <i>Tringa semipalmata semipalmata</i> LA	15556 <i>Tringa semipalmata semipalmata</i> LA 5966 <i>Tringa semipalmata semipalmata</i> NY 47313 <i>Tringa semipalmata semipalmata</i> TX 71706 <i>Tringa semipalmata semipalmata</i> LA 71707 <i>Tringa semipalmata semipalmata</i> LA 71708 <i>Tringa semipalmata semipalmata</i> LA 5967 <i>Tringa semipalmata semipalmata</i> NY
9786 <i>Tringa semipalmata inornata</i> CA	16879 <i>Tringa semipalmata inornata</i> CA 27058 <i>Tringa semipalmata inornata</i> CA 16877 <i>Tringa semipalmata inornata</i> CA
9837 <i>Tringa semipalmata inornata</i> CA	24764 <i>Tringa semipalmata inornata</i> CA
71709 <i>Tringa semipalmata inornata</i> LA	None (Unique sequence)
19407 <i>Tringa semipalmata inornata</i> LA	None (Unique sequence)
43221 <i>Tringa semipalmata semipalmata</i> LA	None (Unique sequence)
5985 <i>Tringa semipalmata semipalmata</i> NY	None (Unique sequence)

**APPENDIX TABLE 9.** ND2 uncorrected pairwise distance values of *Tringa semipalmata semipalmata* and *T. s. inornata* from individuals sampled in Wyoming, California, Florida (*inornata*), New York, Texas (*semipalmata*), and Louisiana (both subspecies). The specimen tissue numbers are row and column names.

	<i>Tringa semipalmata inornata</i>														
	5985	15556	43221	62980	5966	47313	71706	71707	5967	71708	71709	9786	16879	16877	9837
<i>Tringa semipalmata semipalmata</i>															
5985	0.0000	0.0015	0.0030	0.0015	0.0015	0.0015	0.0015	0.0015	0.0015	0.0015	0.0103	0.0088	0.0089	0.0089	0.0117
15556	0.0015	0.0000	0.0020	0.0000	0.0000	0.0000	0.0000	0.0010	0.0010	0.0000	0.0087	0.0077	0.0078	0.0078	0.0077
43221	0.0030	0.0020	0.0000	0.0020	0.0020	0.0020	0.0020	0.0020	0.0030	0.0015	0.0108	0.0098	0.0098	0.0098	0.0098
62980	0.0015	0.0000	0.0020	0.0000	0.0000	0.0000	0.0000	0.0010	0.0010	0.0000	0.0088	0.0078	0.0078	0.0079	0.0078
5966	0.0015	0.0000	0.0020	0.0000	0.0000	0.0000	0.0000	0.0010	0.0010	0.0000	0.0089	0.0079	0.0079	0.0079	0.0079
47313	0.0015	0.0000	0.0020	0.0000	0.0000	0.0000	0.0000	0.0010	0.0010	0.0000	0.0087	0.0077	0.0078	0.0078	0.0077
71706	0.0015	0.0000	0.0020	0.0000	0.0000	0.0000	0.0000	0.0010	0.0010	0.0000	0.0087	0.0077	0.0078	0.0078	0.0077
71707	0.0015	0.0000	0.0020	0.0000	0.0000	0.0000	0.0000	0.0010	0.0010	0.0000	0.0087	0.0077	0.0078	0.0078	0.0077
5967	0.0015	0.0010	0.0030	0.0010	0.0010	0.0010	0.0010	0.0010	0.0000	0.0000	0.0099	0.0089	0.0089	0.0089	0.0089
71708	0.0015	0.0000	0.0015	0.0000	0.0000	0.0000	0.0000	0.0010	0.0000	0.0000	0.0117	0.0102	0.0104	0.0104	0.0102
<i>Tringa semipalmata inornata</i>															
71709	0.0103	0.0087	0.0108	0.0088	0.0089	0.0087	0.0087	0.0087	0.0089	0.0117	0.0000	0.0010	0.0010	0.0010	0.0048
9786	0.0088	0.0077	0.0098	0.0078	0.0079	0.0077	0.0077	0.0077	0.0089	0.0102	0.0010	0.0000	0.0000	0.0000	0.0038
16879	0.0089	0.0078	0.0098	0.0078	0.0079	0.0077	0.0077	0.0078	0.0089	0.0104	0.0010	0.0000	0.0000	0.0000	0.0039
27058	0.0088	0.0077	0.0098	0.0078	0.0079	0.0077	0.0077	0.0078	0.0089	0.0102	0.0010	0.0000	0.0000	0.0000	0.0038
19407	0.0088	0.0068	0.0089	0.0069	0.0070	0.0068	0.0068	0.0078	0.0089	0.0073	0.0039	0.0029	0.0030	0.0030	0.0029
16877	0.0089	0.0078	0.0098	0.0079	0.0079	0.0078	0.0078	0.0078	0.0089	0.0104	0.0010	0.0000	0.0000	0.0000	0.0039
9837	0.0117	0.0077	0.0098	0.0078	0.0079	0.0077	0.0077	0.0077	0.0089	0.0102	0.0048	0.0038	0.0039	0.0039	0.0000
24764	0.0117	0.0077	0.0098	0.0078	0.0079	0.0077	0.0077	0.0077	0.0089	0.0102	0.0048	0.0038	0.0039	0.0039	0.0000

**APPENDIX TABLE 10.** (A) Rotated component matrix and (B) standard deviation and proportion of variance of components from principal components analysis (PCA) of morphological characters of *Tringa semipalmata semipalmata* and *T. s. inornata* sampled in Wyoming (*inornata*), California (*inornata*), Texas (*semipalmata*), and Louisiana (both subspecies).

	PC1	PC2	PC3	PC4	PC5
<b>(A) Trait measured</b>					
Tarsus length	−0.4	−0.2	−0.9	0.0	0.0
Wing chord	−0.9	0.3	0.4	0.0	0.0
Exposed culmen	−0.2	−0.9	0.3	−0.0	−0.0
Depth of distal nares	0.0	−0.0	0.0	1.0	0.0
Ratio of depth of distal nares to exposed culmen	0.0	0.0	0.0	0.0	−1.0
<b>(B) Importance of components</b>					
Standard deviation	9.1	3.7	2.6	0.5	0.0
Proportion of variance	0.8	0.1	0.1	0.0	0.0