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Population genetic structure and species delimitation of a widespread, Neotropical dwarf gecko

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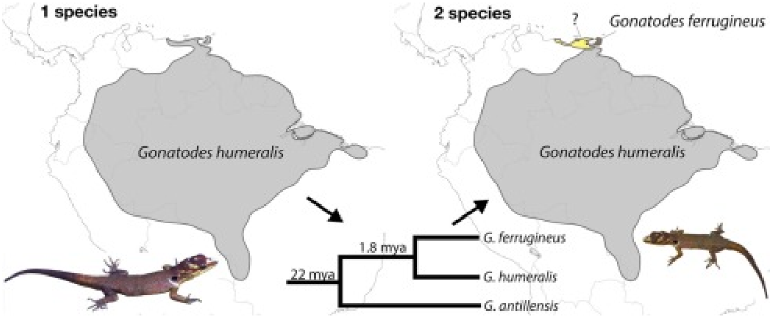
Abstract

Amazonia harbors the greatest biological diversity on Earth. One trend that spans Amazonian taxa is that most taxonomic groups either exhibit broad geographic ranges or small restricted ranges. This is likely because many traits that determine a species range size, such as dispersal ability or body size, are autocorrelated. As such, it is rare to find groups that exhibit both large and small ranges. Once identified, however, these groups provide a powerful system for isolating specific traits that influence species distributions. One group of terrestrial vertebrates, gecko lizards, tends to exhibit small geographic ranges. Despite one exception, this applies to the Neotropical dwarf geckos of the genus *Gonatodes*. This exception, *Gonatodes humeralis*, has a geographic distribution almost 1,000,000 km^2 larger than the combined ranges of its 30 congeners. As the smallest member of its genus and a gecko lizard more generally, *G. humeralis* is an unlikely candidate to be a wide-ranged Amazonian taxon. To test whether or not *G. humeralis* is one or more species, we generated molecular genetic data using restriction-site associated sequencing (RADseq) and traditional Sanger methods for samples from across its range and conducted a phylogeographic study. We conclude that *G. humeralis* is, in fact, a single species across its contiguous range in South America. Thus, *Gonatodes* is a unique clade among Neotropical taxa, containing both wide-ranged and range-restricted taxa, which provides empiricists with a powerful model system to correlate complex species traits and distributions. Additionally, we provide evidence to support species-level divergence of the allopatric population from Trinidad and we resurrect the name *Gonatodes ferrugineus* from synonymy for this population.

Graphical abstract



Keywords

Amazonia, RADseq, Species delimitation, Population genetics, Neotropical phylogeography

1. Introduction

The use of genetic data to study variation among populations and delimit species has provided unprecedented insight into the patterns and processes of speciation (Casillas and Barbadilla, 2017, Domingos et al., 2017, Gratton et al., 2015, Harvey et al., 2017, Lemmon et al., 2012, McKay et al., 2013, Nazareno et al., 2017a, Nazareno et al., 2017b, Weir et al., 2015). Genetic data have been particularly useful in the investigation of poorly-studied taxa from Neotropical regions, such as Amazonia (Angulo and Icochea, 2010, Antonelli et al., 2011). Employing large genetic datasets to Neotropical biogeographic studies can vastly increase their accuracy and resolution relative to previous analyses. Most Neotropical work to date, however, has been conducted using a single type of data (largely mitochondrial data), and has likely led to the oversimplification in our understanding of this biogeographic system (Beheregaray, 2008; Turchetto-Zolet et al., 2013). Thus, in order to elucidate the complex historical scenarios across the Neotropics that have resulted in the immense biodiversity harbored there, studies utilizing larger datasets are needed for a diversity of animal groups.

Several hypotheses have been proposed to explain the historical and spatial patterns of range-limited Amazonian species (see Antonelli et al., 2011, Turchetto-Zolet et al., 2013 for thorough review), nearly all of which depend on the emergence of physical barriers to gene flow that result in allopatric speciation (Haffer, 1969, Haffer, 1997, Endler, 1977, Vanzolini and Williams, 1981, Wallace, 1852). Although there has been considerable debate as to the timing of Amazonian speciation, it now seems clear that cladogenesis has been happening, continually, for tens of millions of years. For instance, many invertebrate, mammal, and bird groups display interspecific divergence between sister species during the Quaternary (<2.6 million years ago [mya]), whereas many amphibians and reptiles exhibit earlier divergence times during the Neogene (>2.6 mya) (Gamble et al., 2008, Antonelli et al., 2011, Fouquet et al., 2015, Turchetto-Zolet et al., 2013). Thus, determining the complex patterns that have generated Amazonian biodiversity may require testing several competing hypotheses and searching for patterns between large- and small-scale studies, across a variety of taxonomic groups. Indeed, and as datasets for Neotropical taxa increase in size, complex historical scenarios have been uncovered that were previously unidentifiable and/or untestable (Alexander et al., 2017, Avila-Pires et al., 2012, Fouquet et al., 2015, Lessa et al., 2003, Nazareno et al., 2017a, Nazareno et al., 2017b, Prates et al., 2016, Werneck et al., 2012).

One trend that molecular genetic data have revealed is that many widely distributed tropical taxa are composed of multiple, often cryptic, species (Funk et al., 2012). These species are usually of smaller body size, with low vagility, and/or those that occupy narrow ecological niches (Camargo et al., 2006, Fouquet et al., 2007b, Wynn and Heyer, 2001). Indeed, even prior to the advent of molecular genetic data, it was predicted that very few widespread nominal taxa in the Neotropics would remain intact upon closer investigation (Lynch, 1979). Subsequently, phylogeographic studies of multiple populations have found that most widespread, non-volant, vertebrate taxa are in fact 'species-complexes' (i.e. composed of multiple undescribed and/or cryptic species). This pattern extends across many terrestrial vertebrate groups including, but not limited to: anole lizards (D'angiolella et al., 2011, Glor et al., 2001), frogs (Camargo et al., 2006, Caminer et al., 2017, Chek et al., 2001, Fouquet et al., 2007a, Fouquet et al., 2014, Funk et al., 2012, Gehara et al., 2014, Guayasamin et al., 2017, Wynn and Heyer, 2001), gecko lizards (Bergmann and Russell, 2007, Gamble et al., 2011a, Geurgas and Rodrigues, 2010, Kronauer et al., 2005), salamanders (Hervas et al., 2016), toads (Fouquet et al., 2007a, Funk et al., 2012, Murphy et al., 2017b), and other herpetofauna (Nunes et al., 2012, De Oliveira et al., 2016). Furthermore, identifying concordant patterns in species' ranges is an important step in the testing of complex biogeographical scenarios that underpin the origins of biodiversity (Clarke et al., 2017a, Clarke et al., 2017b, Costello et al., 2013, Da Silva and Patton, 1993, Díaz-Nieto et al., 2016, Ditchfield, 2000, Gazoni et al., 2018, Gehara et al., 2014, Miralles and Carranza, 2010, Stroud et al., 2017, Turchetto-Zolet et al., 2013).

Whereas many widespread Neotropical taxa appear to be composed of multiple, undescribed species, there are exceptions to this pattern and widely distributed Neotropical taxa do exist. However, these widespread taxa are less frequent than once thought and are typically species that exhibit traits that facilitate high vagility (e.g. being volant, having a large body size, and/or occupying broad ecological niches). Some notable examples of these widespread taxa include: the Amazon Tree Boa (*Corallus hortulanus*), Andersen's Fruit-eating Bat (*Artibeus* anderseni), the Bushmaster (*Lachesis* muta), capybaras (*Hydrochoerus hydrochaeris*), jaguars (*Panthera onca*), the Green Anaconda (*Eunectes murinus*), the Green Iguana(*Iguana iguana*), and the Lesser Treefrog (*Dendropsophus minutus*) (Colston et al., 2013, Ditchfield, 2000, Eizirik et al., 2001, Gehara et al., 2014, Zamudio and Greene, 1997). These examples suggest that range size and abundance of Neotropical species are likely attributable to intrinsic factors such as body size, dispersal ability, and niche breadth, among other traits that have a strong phylogenetic component (Dexter and Chave, 2016, Meiri et al., 2017, Wynn and Heyer, 2001). Thus, some clades are composed mostly of wide-ranging species (large and volant animals), while others are composed mainly of range-limited species (small and dispersal-limited animals). Studying differences in ecological traits and range distribution among these taxa can provide important insights into the patterns and processes responsible for Neotropical biodiversity. However, it is difficult to deduce the relative contribution of individual traits to range size disparities between species, because many traits are autocorrelated at the macroevolutionary scale (Beck and Kitching, 2007, Dexter and Chave, 2016, Hurlbert and White, 2007). Investigating clades that include both geographically widespread and restricted species may provide important insights into how phenotypic differences can influence species distributions (Gehara et al., 2014).

In line with these observations, most Neotropical lizard species have small distributions. However, there are a few notable exceptions, such as the dwarf gecko, *Gonatodes humeralis*, the geographic distribution of which (~7,600,000 km^2) is larger than that of all its congeners combined, by nearly 1,000,000 km^2 (~6,700,000 km^2) (Roll et al., 2017). *Gonatodes humeralis* occurs across Amazonia and the Guiana Shield, as well as in forested enclaves and gallery forests in the adjacent Cerrado and Caatinga biomes, and on the island of Trinidad (Avila-Pires, 1995, Murphy, 1997, Ribeiro-Júnior, 2015, Roberto et al., 2014, Vanzolini, 1955). Overall, its current distribution occupies a geographic area marginally smaller than that of the continental United States and overlaps with 13 currently described congeneric species (Supplemental Fig. 1). *Gonatodes humeralis* also exhibits a broad niche breadth, occurring in a variety of habitat types including: primary and secondary forest, riparian forest, gallery forest, forest edges, bamboo forest, and human dwellings (Carvalho et al., 2008, Dixon and Soini, 1986, Higham et al., 2017, Hoogmoed, 1973, Vanzolini and Williams, 1981, Vitt and Zani, 1996, Vitt et al., 1997, Vitt et al., 2000). Its massive distribution and extensive niche breadth contrast with those of its congeners, most of which occupy specialized niches with small, distributions in Central and South America and several islands of the Lesser Antilles (Supplemental Fig. 1). In the context of recent discoveries suggesting that widespread Neotropical taxa are uncommon, the diminutive *G. humeralis* (maximum 41.5 mm snout-vent length; Avila-Pires, 1995) is an unlikely candidate for being a single species. However, if *G. humeralis* is, in fact, one widespread species, then *Gonatodes* harbors both widespread and geographically restricted taxa, providing a powerful model system for identifying traits that may influence species distributions.

Previous investigations on *G. humeralis* have revealed evidence for genetic, ecological, and morphological variation between populations across its range (Avila-Pires, 1995, Avila-Pires et al., 2012, Rivero-Blanco, 1979, Vitt et al., 1997), and early hypotheses suggested that populations should exhibit relatively shallow divergence times, within the Pleistocene (Vanzolini and Williams, 1981, Vitt et al., 1997). Supporting this, the first multi-locus phylogenetic analysis of *Gonatodes* revealed that *G. humeralis* samples from eastern and western Amazonia likely shared a common ancestor in the late Pliocene or early/mid Pleistocene, approximately 1.9 (1.1–2.7) mya (Gamble et al., 2008). Later, the most comprehensive phylogeographic analysis to date investigated the history of *G. humeralis* populations in eastern Amazonia using two mitochondrial markers (Cytb & 16S) from 56 individuals (Avila-Pires et al., 2012). The authors found little phylogenetic resolution among sampled populations and no evidence that Amazonian rivers (namely, the Amazon and Tocantins) have acted as isolating mechanisms between sampled populations in eastern Amazonia. The authors concluded that range-wide sampling and the addition of nuclear markers would be necessary to obtain sufficient resolution of any phylogeographic hypothesis relating to this species.

We herein investigate the geographically widespread gecko, *G. humeralis,*across its range in northern South America and Trinidad. Specifically, we test two alternative hypotheses: (i) if *G. humeralis* is typical of most small, non-volant Neotropical vertebrates, we expect to uncover a species-complex composed of multiple cryptic, or morphologically similar, species; (ii) conversely, if *G. humeralis* is an atypical taxon, then we expect it to be a single, widespread species across its contiguous Amazonian range, and potentially also on the island of Trinidad. To test this, we generated restriction-site associated DNA sequencing (RADseq) data, and a multi-locus Sanger-sequenced dataset using traditionally informative nuclear and mitochondrial markers. We began by investigating the population genetic structure of *G. humeralis* across its range, then reconstructed the relationships of those alleles between populations, and used these relationships to generate specific species delimitation hypotheses for further testing. Indeed, we predicted that *G. humeralis*would consist of multiple, cryptic species with distributions comparable in size to those of other species of *Gonatodes*. However, we found that *G. humeralis* is a single, widespread species across Amazonia, whereas the population on the island of Trinidad appears to be highly divergent and independently-evolving. We discuss these results in a comparative context with other Neotropical species and posit that this genus of geckos (*Gonatodes*) may yield unprecedented insights into the origins and maintenance of Neotropical biodiversity.

2. Materials and methods

2.1. Sampling

We sampled 31 individuals of *G. humeralis* from 13 localities across its range (Fig. 1). Three individuals of *G. antillensis* were included as an outgroup (Russell et al., 2015). We extracted genomic DNA for downstream genetic sequencing from tail clips or liver, using the Qiagen® DNeasy Blood and Tissue extraction kit.

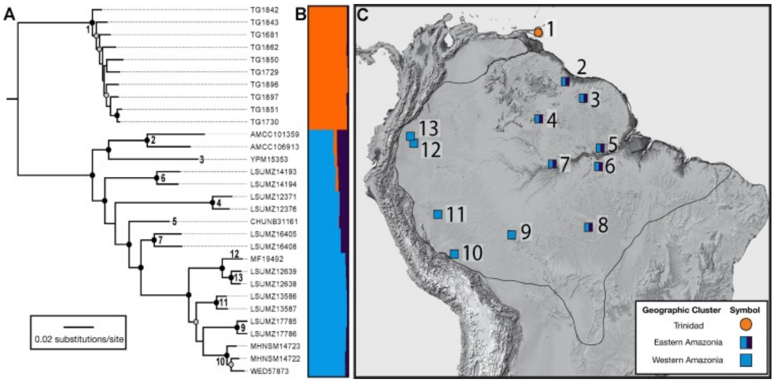


Fig. 1. (A) Maximum-likelihood tree computed using 22,486 unlinked SNPs executed in RAxML, bootstrap values ≥70 reported (bootstrap values of 100 = black circles, bootstrap values from 70 to 99 = gray circles). Bolded numbers correlate individual or clade with sampling locality depicted on map. (B) Distruct plot depicting proportions of shared alleles present in the *G. humeralis* lineage determined by STRUCTURE analysis, K = 3 (Supplemental Fig. 2). (C) Map indicating sampling localities, within the geographic range of *G. humeralis*, in relation to cluster assignments (Trinidad = circle, west Amazonia = solid square, east Amazonia = patterned square) in relation to their geographic locality (Supplemental Table 1). Further, locality 8 (represented by CHUNB47049) is absent from the RADseq tree in panel A (see Methods).

2.2. RADseq data

We generated a reduced-representation genomic dataset for all *G. humeralis* individuals using restriction-site associated DNA sequencing(RADseq). RADseq libraries were constructed following a protocol modified from Etter et al. (2011), as described by Gamble et al. (2015). Briefly, genomic DNA was digested using high-fidelity *SbfI* restriction enzyme (New England Biolabs). We ligated individually barcoded P1 adapters onto the *SbfI* cut site for each sample. Samples were pooled into multiple libraries, sonicated, and size selected into 200- to 500-basepair (bp) fragments using magnetic beads in a PEG/NaCl buffer (Rohland and Reich, 2012). Libraries were blunt-end-repaired and dA tailed. To each of the pooled libraries, we ligated a P2 adapter containing unique Illumina barcodes. Libraries were amplified using 16 PCR cycles with Phusion high-fidelity DNA polymerase (New England Biolabs), and were size-selected a second time into 250- to 600 bp fragments using magnetic beads in PEG/NaCl buffer. Libraries were sequenced using paired-end 125 bp reads on the Illumina®

HiSeq2500 at the Institute for Integrative Genome Biology, University of California, Riverside. RADseq data for the 10 individuals from Trinidad were previously published (Gamble et al., 2018).

We trimmed and demultiplexed raw single-end Illumina sequencingreads by their individual-specific barcodes using the process_radtags command in STACKS [v1.23]; (Catchen et al., 2011). After the removal of low-quality reads, restriction site overhangs, and barcodes, the 3′ ends of the 125 bp reads were trimmed to 100 bp. Cleaned reads were imported into the PyRAD pipeline [v3.0.63] for *de novo* assembly [steps 2–7] (Eaton, 2014). One individual, CHUNB47049, was removed from the RADseq dataset prior to filtering, due to low-quality reads (adjusting our RADseq dataset, N = 30). This removed locality #8 (Fig. 1c) from all RADseq data analyses. We assayed various filtering criteria configurations, including varying the minimum read depth per locus from 4 to 12; maximum number of "N"s per locus from 4 to 6; within- and across-sample clustering threshold from 80 to 98%; and the minimum number of individuals with sequence data for a locus needed from 10 to 28. To obtain a dataset with >10,000 and <50,000-unlinked markers incorporating ≤10% missing data, we set the final filtering criteria for exclusion of any locus with a read depth of less than 8 reads, and missing data ("N" characters) to ≥5. We set the within- and across-sample clustering threshold to 95% sequence identity, and the minimum number of individuals required for data to be included in a final locus was set to 25 of the 30 individuals. All other PyRAD parameters used default settings. The final dataset consisted of 35,260 informative loci with 67,173 total single-nucleotide polymorphisms (SNPs), 26,486 of which were unlinked (sampling only one SNP per RAD locus). We subsampled and reformatted this final dataset for all downstream RADseq data analyses; further data specifics for each analysis are provided below.

2.3. Sanger sequence data

We also produced sequence data from fragments of six molecular markers using Sanger sequencing of PCR amplicons. This consisted of four nuclear genes: microtubule-associated protein 1b – exon 5 (MAP1b), recombination-activating gene 1 (RAG1), oocyte-maturation factor MOS (CMOS), and protein tyrosine phosphatase nonreceptor type 12 (PTPN12); and two mitochondrial genes: NADH dehydrogenase subunit 2 (ND2) and 16S ribosomal subunit (16S). PCR conditions and primer sequences are described elsewhere: MAP1b (Werneck et al., 2012), RAG1, CMOS, PTPN12 (Gamble et al., 2011b), ND2 (Jackman et al., 2008), and 16S (Gamble et al., 2008). We Sanger-sequenced PCR amplicons using GeneWiz® single-pass sequencing, then assembled and quality-trimmed raw sequences using Geneious® [v9.1.5] (Kearse et al., 2012). GenBankaccession numbers for all sequences are listed in Supplemental Table 1. Sequences were aligned using MUSCLE [v3.8.425] (Edgar, 2004) and alignments refined by eye, if necessary. Models of molecular evolutionwere chosen based on AICc and BIC criteria, computed using MEGA7 (Kumar et al., 2016).

2.4. Population genetic analyses

We visualized the population-level genetic diversity within *G. humeralis sensu lato* and estimated the number of genetic populations in Hardy-Weinberg equilibrium present in our RADseq data using STRUCTURE [v2.3.4] (Pritchard et al., 2007). We investigated possible values of K (where K is equal to the number of populations of alleles) between 1 and 6 with a subset of the unlinked SNP data, using only the first 16,382 SNPs, for computational efficiency, with the admixture model (starting alpha = 1.0), with correlated allele frequencies (fixed lambda = 1.0), and all other priors set to default. We tested K values by repeating five independent MCMC chains of 150,000 replicates, each with a 10% burnin. STRUCTURE output was parsed and visualized using the Evanno method in Structure Harvester (Earl and vonHoldt, 2011, Evanno et al., 2005) and the CLUMPAK server (Kopelman et al., 2015).

To further characterize the population genetic structure of mainland *G. humeralis* and how this structure might confound our species delimitation methodologies (see *Species Delimitation* below), we tested for (i) isolation-by-distance (IBD), (ii) deviations from neutral expectations, and (iii) calculated metrics of genetic diversity. (i) We tested for isolation-by-distance (IBD) using Mantel's test (Diniz-Filho et al., 2013, Mantel, 1967). We generated a geographic distance matrix from locality information using the Geographic Distance Matrix Generator software (Ersts, 2006) and a pairwise F_{st} distance matrix for our unlinked SNP (26,486) dataset using Arlequin [v3.5.2.2] (Excoffier and Lischer, 2010). We converted the geographic distance into a Euclidean distance matrix with the *quasieuclid* function in the ade4 package [v1.7.4] (Dray and Dufour, 2007) in R (R Core Team, 2016). We conducted Mantel's test, also using ade4, with the *mantel.randtest* function, creating 999 randomized permutations to calculate p-values. (ii) We tested whether sampled populations deviated from expectations under a neutral model by calculating Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) statistics for two datasets, our concatenated mitochondrial loci (mtDNA) and RADseq SNPs. Neutrality test statistics for the mitochondrial data were estimated using DNAsp [v5.0]; (Librado and Rozas, 2009) and for genotypic SNP data we used PopGenome [v2.1.6] package (Pfeifer et al., 2014) in R (R Core Team, 2016). (iii) We calculated nucleotide diversity (π) and within- and between-group genetic distances for ND2 for all three populations and their sister group, *G. antillensis*, in DNAsp [v5.0] (Librado and Rozas, 2009) and MEGA7 (Kumar et al., 2016), respectively. In addition, we calculated net between-group distances (Nei and Li, 1979) between *G. humeralis* clusters, as identified by STRUCTURE, using MEGA7, for 16S and ND2 separately, using uncorrected p-distances (Edwards and Beerli, 2000). Standard error estimates were calculated using 500 bootstrap replicates.

2.5. Phylogenetic inference

We estimated the phylogenetic relationships among sampled *G. humeralis* using maximum-likelihood (ML) and Bayesian methods. To analyze our data in an ML framework, we formatted the 26,486 unlinked RADseq SNPs using the shinyPhrynomics package [v1.3] (Leaché et al., 2015) based in R (R Core Team, 2016). We generated a ML tree using RAxML [HPC–v8.2.9] under a GTR + Γ model with 1000 rapid bootstrap replicates, using the automatic bootstopping function (Stamatakis, 2014), implemented on the CIPRES cluster (Miller et al., 2010). We corrected for SNP-only data biases by estimating ML branch lengths from SNP-only data using the Stamatakis correction, which focuses on minimizing branch length overestimation due to acquisition bias, as described for use with SNP data by Leaché et al. (2015).

We also produced a rooted mitochondrial gene tree in a Bayesian framework, to compare with the nuclear SNP data tree, using BEAST2 [v2.5.1] under a strict clock (Bouckaert et al., 2014) on the CIPRES cluster (Miller et al., 2010). The concatenated mitochondrial (mtDNA) data (ND2 and 16S) consisted of 34 samples, including three *G. antillensis*, for a total of 1484 bp. We used the GTR + Γ model and a Yule tree prior with 5×10^8MCMC iterations with a 10% burnin. Bayesian analyses were replicated three times and examined by eye using Tracer [v1.6.1] to ensure convergence. Post-burnin trees from all three runs were combined to estimate final tree parameters using Log Combiner and Tree Annotator, respectively.

Next, we estimated divergence time among *G. humeralis* populations using the StarBEAST2 [v0.15.1] (Ogilvie et al., 2017) module of BEAST2 (Bouckaert et al., 2014). We used the multi-locus Sanger sequence data, sampling 15 *Gonatodes* species using a secondary calibration at the root following Higham et al. (2017) and individuals of three *G. humeralis*phylogeographic clusters identified by STRUCTURE: Trinidad, eastern, and western Amazonia, based on the (see *Population Genetic Analyses*). The final dataset used in this analysis included seven loci: ACM4, CMOS, mtDNA (ND2 + 16S), PDC (phosducin), PTPN12, RAG1, and RAG2; nuclear loci were phased using DNAsp [v5.0] (Librado and Rozas, 2009, Stephens et al., 2001). Loci used in this analysis were chosen specifically to minimize the amount of missing data per taxon while combining newly generated and previously published sequence data (Supplemental Table 2). Indeed, each locus was provided its own best-fit as calculated in MEGA7 (and has an available model in StarBEAST2), this was HKY + Γ for all nuclear loci and GTR + Γ for our concatenated mtDNA genes. We used an uncorrelated lognormal clock model, with secondary calibration from a previously published fossil-calibrated phylogenetic reconstruction, to provide a prior on the root age between *Gonatodes* and its sister clade *Lepidoblepharis* at approximately 72.5 (±7.5) mya, with a uniform distribution to reflect confidence intervals (Gamble et al., 2015).

To corroborate these findings, we utilized the published rate of molecular evolution for the mitochondrial ND2 gene in geckos. We estimated the divergence time between the mainland and Trinidad using p-distances assuming a strict molecular clock. We calculated p-distances in MEGA7 (Kumar et al., 2016) and calculated the divergence time according to the previously published rate of molecular evolution for the ND2 locus in geckos, at 0.57% (per lineage rate) per million years (Macey et al., 1999), i.e. (p-distance/2 * 100) * 0.57 = lineage-divergence in millions of years.

2.6. Species delimitation

We assessed whether *G. humeralis* consists of one, two, or three putative species using our phylogenetic and STRUCTURE results to guide assignment of individuals into putative species-level lineages using three species delimitation methods: Poisson Tree Processes (PTP), STACEY, and Bayes Factor Delimitation (BFD).

First, we analyzed species boundaries using our Bayesian mitochondrial gene tree with the single-rate PTP test, using the PTP web service (http://mptp.h-its.org/#/tree), with the p-value set at 0.001 (Kapli et al., 2017).

Second, we used STACEY [v1.2.4] (Jones, 2017) with our Sanger sequenced dataset (CMOS, MAP1b, PTPN12, RAG1, and mtDNA), including *G. antillensis* and *G. concinnatus* as outgroups. In accordance with program documentation and additional specifications outlined by Barley et al. (2018), we provided an exponential distribution with a mean of 0.1 for the “popPriorScale” parameter, a lognormal distribution with a mean of 5 and a standard deviation of 2 to the “bdcGrowthRate” prior, and the “collapseWeight” was provided a uniform distribution with the lower and upper bounds set at 0 and 1, respectively (Barley et al., 2018). In addition, each gene partition was provided the best-fit model of molecular evolution used by the STACEY package (CMOS and PTPN12 – JC; MAP1b and RAG1 – HKY; mtDNA – TN93), an independent strict molecular clock, with rate priors calculated from a log-normal distribution that were given a mean of 0 and standard deviation of 1 (Barley et al., 2018). We ran three independent chains of 5.0×10^7 MCMC repetitions, sampling every 5000 trees, and compared trace files using Tracer [v1.7] (Rambaut et al., 2018). We combined tree files using LogCombiner, visualized them using DensiTree, and analyzed the resulting 30,000 trees using the SpeciesDelimitationAnalyzer [v1.8], herein STACEY and SpeciesDelimitationAnalyzer are referred to as SSDA. We used a burnin of 5000 trees and a collapse-height of 0.0001 to calculate our final species delimitation posterior.

Third, we compared two alternative species models, the 2-taxon (PTP: Trinidad/mainland) and 3-taxon models (SSDA: Trinidad/east Amazonia/west Amazonia), using BFD with the RADseq SNP dataset (Leaché et al., 2014). BFD utilizes the path-sampling analysis of the SNAPP package (Bryant et al., 2012) in BEAST2 (Bouckaert et al., 2014) to infer species boundaries directly from biallelic SNP data by comparing the likelihood of two differing species models using Bayes factors (Leaché et al., 2014). We used 48 path sampler steps with 100,000 MCMC repetitions and a 10% burnin to sample from 500,000 MCMC SNAPP replications. We systematically compared models using Bayes factors, calculated using $BF = 2 * (|\text{model } 1| - |\text{model } 2|)$, where the “model” represents the marginal-likelihood estimate from the specific model being compared against (Ogilvie and Leaché, 2016). We ensured that each model was better than random by estimating the marginal-likelihood for a 3-taxon model, where all individuals were randomly assigned to a “species” to ascertain that both models were better than an unrealistic “null” model (Burbrink et al., 2011).

Lastly, we conducted topology tests to assess whether we could reject the hypothesis that eastern and western Amazonia were reciprocally monophyletic, potentially providing support for the hypothesis that each cluster is a distinct lineage. We constructed two sets of ML trees using RAxML [HPC2–v8.2.10] under a GTR + Γ model, with RAxML’s automatic bootstopping function (Stamatakis, 2014), also implemented on the CIPRES cluster (Miller et al., 2010) for our RADseq SNP dataset (described above) and for our mtDNA (ND2 and 16S). We constructed an unconstrained tree and a tree for which we enforced a reciprocal monophyletic constraint between eastern and western Amazonia. We conducted topology tests between both trees using the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) and Shimodaira’s Approximately Unbiased (AU) test (Shimodaira, 2002) in a likelihood framework under a GTR model with an estimated rate matrix. Topology tests were conducted in Phylogenetic Analysis Using Parsimony (PAUP*) [v 4.0a157] (Swofford, 2002). We calculated significance using 10,000 RELL bootstrap replications.

3. Results

3.1. Population genetic STRUCTURE

The best-fit model for the STRUCTURE analysis was for three populations of alleles in Hardy-Weinberg equilibrium (K = 3). These STRUCTURE results in light of phylogenetic reconstruction indicated that, Trinidadian individuals are distinct from the mainland, but most alleles are shared across the mainland. However, there is a small proportion of unique alleles specific to eastern Amazonia (Fig. 1, Supplemental Fig. 2), which could be due to a variety of factors (see Discussion). Alleles belonging to the allopatric Trinidad population were distinct from those of the mainland ('orange') (Fig. 1, Table 1), so we excluded Trinidadian individuals from certain subsequent population-level analyses (i.e. neutrality tests and testing for IBD). Further investigation into the population structure and demographic history of mainland *G. humeralis* involved three analyses. (i) we tested against a neutral model of molecular evolution for evidence of rapid population expansion across the mainland, and we looked for concordance between two test statistics, Tajima's D and Fu's Fs. Neither test showed a deviation from neutrality for either the mitochondrial or RADseq SNP data (Table 3). (ii) we tested for the presence of IBD across the mainland using Mantel's test (Table 1, Table 2) by correlaing a matrix of pairwise genetic distances and a matrix of geographic distances. This analysis revealed strong evidence for IBD across mainland South America (Table 2, $R^2 = 0.637$, p-value = 0.001). (iii) we estimated within-population genetic distance (p-distance) and within-population nucleotide diversity (π) for each population and the outgroup, *G. antillensis*, for mtDNA (Supplemental Table 3). These measurements showed that *G. humeralis*from eastern Amazonia exhibits more genetic diversity than western populations, and that Trinidadian *G. humeralis* display very little genetic diversity overall when compared to mainland populations.

Table 1. Pairwise uncorrected net between-group mean p-distances for mitochondrial data: ND2 (below diagonal) and 16S (above diagonal). Distances and confidence intervals calculated via 500 bootstrap replicates using MEGA7 software (Kumar et al., 2016).

Population	East	West	Trinidad	Outgroup
East	0	0.012 ± 0.003	0.02 ± 0.006	0.25 ± 0.021
West	0.05 ± 0.01	0	0.023 ± 0.006	0.249 ± 0.021
Trinidad	0.1 ± 0.01	0.09 ± 0.01	0	0.253 ± 0.021
Outgroup	0.56 ± 0.01	0.56 ± 0.02	0.56 ± 0.01	0

Table 2. Summary of test results sectioned by phylogeographic cluster. Mantel test reports indicate within and across cluster presence of isolation by distance (*** indicates significant correlation). Test statistics reported within and across clusters indicate divergence from a neutral model (no tests reported as being significant); "mtDNA" tests were conducted in DNAsp [v5.0]; (Librado and Rozas, 2009), whereas "RADseq" tests were conducted in R (R Core Team, 2016) using the PopGenome [v2.1.6] package (Pfeifer et al., 2014). Species delimitation method results are reported by geographic cluster; (✓) indicates the delimitation of that cluster as a separate species via the method listed, whereas (–) indicates a failure to delimit a geographic cluster as a species (PTP – Poisson Tree Processes; SSDA – STACEY and SpeciesDelimitationAnalyzer; BFD – Bayes Factor Delimitation).

Geographic Cluster	Mantel's Test		Neutrality Test			Species Delimitation		
	R-square	P-value	Data	Tajima's D	Fu's Fs	PTP	SSDA	BFD
Trinidad	−0.504	0.794	mtDNA	−0.036	−1.910	✓	✓	✓
			RADseq	−1.439	−0.905			

Mainland	0.637	0.001^{***}	mtDNA	−0.942	0.579	✓	✓	✓
			RADseq	−1.972	−0.978			
Mainland (East)	0.045	0.386	mtDNA	−0.796	−0.241	–	✓	✓
			RADseq	−1.314	1.192			
Mainland (West)	0.594	0.162	mtDNA	0.148	4.142	–	✓	✓
			RADseq	−0.469	0.143			

Table 3. Species delimitation models compared using Bayes factors with BFD, ranked by marginal likelihood estimate (MLE). Bayes factors reported as pairwise comparisons of a randomized 3-taxon model versus being listed by each model [Bayes factor = 2 * (|MLE model 1| − |MLE model 2|)]. Pairwise ln(BF) calculations select both the 2-taxon (10.4) and 3-taxon (10.8) models as being significantly better than random species assignments using the Kass and Raftery (1995) scale; where ln(BF) ≥ 5 there is strong support for the model with the higher MLE. Pairwise comparison between 2-taxon and 3-taxon models results in a ln(BF) = 9.5, providing decisive support in favor of the 3-taxon model.

Taxon Statement Model Tested	**MLE**	**Rank**	**Bayes Factor**
Randomized 3-taxon Statement	|−104081.08|	3	–
2-taxa (Trinidad & Mainland)	|−87439.54|	2	16645.86
3-taxa (Trinidad, East, & West)	|−80789.26|	1	6645.96

3.2. Phylogenetic inference

Phylogenetic relationships at well-resolved nodes was largely concordant across the methodologies and data sets used (Fig. 1, Fig. 2b). ML and Bayesian methods recovered reciprocally monophyletic Trinidadian and mainland populations using RADseq and Sanger sequenced mitochondrial and nuclear datasets (Fig. 1, Fig. 2, Supplemental Figs. 3, 4, and 5). Indeed, overall relationships among mainland populations were concordant at well-supported nodes, with a broader Amazonian clade containing a nested monophyletic group from western Amazonia. Between-group mean genetic distances among *G. humeralis*phylogeographic clusters ranged from 0.05–0.1 and 0.012–0.023 for ND2 and 16S, respectively (Table 1). Divergence times between Trinidad and mainland *G. humeralis* lineages were estimated to occur in the early Pleistocene: 1.89 mya [0.90–2.42, 95% HPD] (Fig. 2; Supplemental Fig. 5) using a secondary calibration and 2.7 mya [2.45–2.91] assuming a strict clock using the published ND2 rate calibration in geckos [p-distance = 0.094 ± 0.008]. There was more consensus on the estimated divergence time between populations in eastern and western Amazonia, where mean values varied from 1.59 [0.13–3.0] (calibration) to 1.60 [1.48–1.71] mya (ND2 rate) [p-distance = 0.056 ± 0.004].

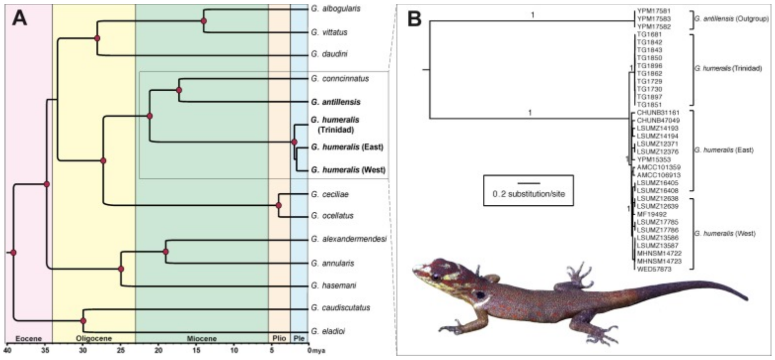


Fig. 2. Phylogenetic inference using two Bayesian inference methods. (A) Time-calibrated StarBEAST2 multi-locus phylogenetic inference (trimmed from Supplemental Fig. 5). Red dots at nodes indicate nodal support ≥0.95 posterior probability. Scale in millions of years before present (mya) and geological era indicated via shaded boxes (Plio = Pliocene, Ple = Pleistocene). (B) Mitochondrial gene tree generated with ND2 and 16S on zoomed in region from part A. Numeric values indicate posterior probability support for the adjacent node. Shallow, haplotype-level support values are removed for clarity. Precise posterior support for all nodes, however, are

reported in cladogram format in Supplemental Fig. 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Species delimitation

We utilized three well-documented statistical species delimitation methods (PTP, STACEY, BFD) to examine species limits between the three phylogeographic clusters previously identified by STRUCTURE (Fig. 1). Analysis of our mtDNA gene tree using PTP revealed significant species-level divergence between Trinidad and mainland clades (p-value = 0.001), but not between eastern and western Amazonia (Supplemental Fig. 3). Analysis of the multi-locus Sanger sequenced dataset with STACEY and SpeciesDA (SSDA) supported the Trinidad and mainland South American clades as being distinct, species-level lineages (pp = 0.999) (Table 2, Supplemental Fig. 4). SSDA analyses also yielded an additional species delimitation hypothesis within the mainland, identifying populations from eastern Amazonia and western Amazonia as separate species (Table 2, Supplemental Fig. 6). We used BFD to compare the two-species (Trinidad + mainland) model, favored by PTP, and the three-species (Trinidad + eastern Amazonia + western Amazonia) model, favored by SSDA, using our RADseq data in a coalescent framework. Pairwise Bayes Factors (BF) calculations selected both the 2-taxa [ln(BF) = 10.4] and 3-taxa [ln(BF) = 10.8] models as being significantly better than random species assignments using the Kass and Raftery (1995) scale; if ln(BF) ≥ 5 there is strong support for the model with the higher MLE. The pairwise comparison between 2-taxon (PTP) and 3-taxon (SSDA) models provided stronger support for the 3-taxon model [ln(BF) = 9.5] (Table 3, Supplemental Fig. 4). To further examine the feasibility that *G. humeralis* from eastern and western Amazonia belong to distinct species, we tested whether our data supported reciprocal monophyly between the populations using topology tests by generating constraint trees for each dataset (trees not shown). Indeed, both SH and AU tests rejected the hypothesis that eastern and western Amazonian populations are reciprocally monophyletic, using the RADseq SNP data (SH p-value <0.0001, AU p-value ~0) and mtDNA data (SH p-value = 0.0055, AU p-value = 0.0006).

4. Discussion

Phylogenetic analyses recovered *G. humeralis* populations from Trinidad as sister to mainland populations, with a western Amazonian cladenested within populations from eastern Amazonia (Fig. 1, Fig. 2b). Furthermore, STRUCTURE analysis inferred three populations of alleles in Hardy-Weinberg equilibrium (K = 3), with no individuals belonging purely to the third “ghost” population (‘purple’). This STRUCTURE pattern can be the result from two scenarios (Lawson et al., 2018): (1) admixture with an extinct/unsampled population or (2) genetic diversityin eastern Amazonia that did not establish in western populations, potentially through isolation-by-distance (IBD) mediated gene flow or a population bottleneck during stepwise westward range expansion. Distinguishing between scenarios (1) and (2) is difficult and they are not mutually exclusive. At present, testing for admixture, scenario 1, is not possible with our current sampling as individuals from the putative “ghost” population are also needed. It’s possible that increased sampling across the Guiana Shield could identify *G. humeralis* populations that harbor an increased frequency of these “ghost alleles”. Indeed, population differentiation in this region has been noted previously for other taxa (Noonan and Gaucher, 2005). However, we posit (2) is a more likely scenario, i.e. extensive genetic diversity specific to eastern Amazonian populations, for three reasons: (i) we found much greater genetic diversity in eastern Amazonia (Supplemental Table 3) and little evidence for shared mtDNA haplotypes between localities, as did Avila-Pires et al (2012), which would be expected under this scenario; (ii) we recovered a signal of IBD across the mainland, which could account for the eastern specificity of these alleles via dropout; and (iii) western Amazonian populations are monophyletic, which would be expected if there were a population bottleneck during westward colonization. However, apart from weighing these lines of evidence, the current state of knowledge and our current sampling provide no definitive way of differentiating them. Thus, future work may warrant further examination of these possibilities.

Our phylogenetic and STRUCTURE results informed the possibility that Trinidadian divergence from the mainland is sufficient to warrant taxonomic reevaluation. Examining species limits using multiple methods and data types consistently identified the Trinidad populations as distinct species from the mainland populations, while a subset of analyses (SSDA & BFD) further split populations from eastern and western Amazonia. We first address whether the Trinidad populations represent a distinct species from the mainland populations, and then discuss whether the South American populations consist of one or more species.

All species delimitation analyses recovered Trinidadian populations as being distinct from Amazonian *G. humeralis* (Table 2; Supplemental Figs. 3 and 4). Additionally, uncorrected genetic distances in mitochondrial ND2 (10%) between Trinidad and eastern populations (Table 1) are comparable to mitochondrial genetic distances among other recognized sister species of geckos, which typically range from 4.1% to 35.5% (Botov et al., 2015, Grismer et al., 2014a, Grismer et al., 2014b, Grismer et al., 2017, Oliver et al., 2007, Pepper et al., 2006, Portik et al., 2013). Although species delimitation based solely on pre-determined sequence divergence values is difficult, if not impossible, to justify due to variations in effective population sizes and lineage-specific substitution rates (Barraclough et al., 2009, Moritz and Cicero, 2004, Pons et al., 2006), genetic distances among putative taxa can highlight taxa that warrant closer examination using other species delimitation methodologies (Gamble et al., 2012a, Hickerson et al., 2006), e.g. PTP, SSDA, and BFD. Thus, the bulk of the evidence supports recognition of the Trinidadian population as an independently evolving metapopulation lineage, or species (de Queiroz, 2007), distinct from mainland *G. humeralis*. Because the type locality of *G. humeralis* is from Peru (Guichenot, 1855, Rivero-Blanco, 1979), mainland South American populations should retain that name. Geckos on Trinidad, however, were previously described as *G. ferrugineus* (Cope, 1864) and we resurrect that name from synonymy for the Trinidadian population and briefly discuss its unusual nominal history.

Gonatodes ferrugineus has a complex taxonomic history (see supplement for complete synonymy). Cope (1864) described *G. ferrugineus* from material collected on Trinidad that Theodore Gill deposited in the Smithsonian. Although the original description was ambiguous, and the type presumably lost (Rivero-Blanco, 1979), Cope (1868) later identified a *G. ferrugineus* specimen (presumably being unaware of *G. humeralis*) among a collection of lizards from Peru and thus later naturalists assumed that *G. ferrugineus* was morphologically similar-to, and perhaps a junior synonym of, *G. humeralis* (Guichenot, 1855). *Gonatodes ferrugineus*was eventually synonymized with *G. humeralis,* although no justification was provided for the decision (Donoso-Barros, 1968). However, throughout the late 19th and most of the 20th centuries discrepancies in nomenclature were apparent. Some herpetologists appeared to be unaware of *G. ferrugineus* and listed *G. humeralis* as occurring on Trinidad, likely based on their own experiences with this species while working in South America (Parker, 1935, Roux, 1926). Others listed *G. ferrugineus* as occurring on Trinidad and *G. humeralis* on the mainland (Boulenger, 1885, Burt & Burt, 1933). Wermuth (1965) added to the confusion by indicating that both *G. ferrugineus* and *G. humeralis* co-occur on Trinidad. However, following the explicit synonymy of Donoso-Barros (1968) and Rivero-Blanco's thorough scholarly review (1979), synonymy of *G. ferrugineus* with *G. humeralis* was unanimously accepted (Avila-Pires, 1995, Kluge, 1993, Kluge, 1995, Kluge, 2001).

Gonatodes ferrugineus is currently morphologically indistinguishable from *G. humeralis* although there appear to be some qualitative differences in proportionality of the face, body size, and coloration in adult males that may, upon further investigation, diagnose this species (Authors' pers. obs.; Rivero-Blanco, 1979). Coloration may be particularly useful as adult males from Trinidad are generally not as colorful as those from mainland South America (Supplemental Fig. 1). Trinidadian males lack red spots on the sides of the body and their heads tend to favor orange/yellow rather than red and white/blue, both of which are typical features of most South American populations (Authors' pers. obs.; Rivero-Blanco, 1979). Similarly-colored males to those from Trinidad have also been observed in northern Venezuela (Rivero-Blanco, 1979), leading to the possibility that *G. ferrugineus* occurs there as well (Supplemental Fig. 1). Indeed, several Trinidadian endemics exhibit distributions that extend into northern Venezuela, such as *Gonatodes ceciliae, Gonatodes vittatus*, *Polychrus auduboni,* and *Flectonotus* *fitzgeraldi*(Murphy, 1997, Murphy et al., 2017a). Further, previous studies that have examined morphological variation within *G. humeralis* have not included specimens from Trinidad (Avila-Pires, 1995, Avila-Pires et al., 2012, Vitt et al., 1997). Thus, future work should attempt to identify diagnostic phenotypic differences to complement the identified genotypic characters between these two species and determine the geographical boundaries of these species (Supplemental Fig. 1). It is worth pointing out a gap in our sampling from the northern Guiana Shield to Trinidad. Indeed, having not sampled Venezuelan populations may confound species delimitation metrics. However, we find this unlikely as we see no evidence of gene flow between Trinidad andthe mainland, even when K = 2 (Supplemental Fig. 2) and 9.4% pairwise divergence at the mitochondrial locus ND2 is considerable, and likely reflects substantial reproductive isolation.

Although *G. ferrugineus* was revealed to be unambiguously distinct from mainland populations in all analyses, the status among South American populations was less straightforward. SSDA and BFD both provided support for a species delimitation model that splits mainland *G. humeralis* into two species, occupying eastern and western Amazonia (Table 2, Table 3, Supplemental Fig. 4). This hypothesis was bolstered by the fact that western Amazonia did not possess a large proportion of eastern-specific alleles (Fig. 1b) and that western Amazonia is monophyletic, although not reciprocally monophyletic with relation to eastern populations (Fig. 1a). These data are also congruent with previous work showing that the western Amazonian populations exhibit ecological differences compared to eastern populations. Namely, eastern *G. humeralis* occurs in primary forest, whereas western *G. humeralis* occur frequently in clearings, secondary forests, and human dwellings (Vitt et al., 1997). Additionally, a model that supports a parapatric mode of speciation across Amazonia would support the gradient hypothesis of Amazonian biogeography (Endler, 1977). However, there is emerging evidence that intraspecific, population-level processes can confound assumptions made by coalescent species delimitation methods, such as SSDA and BFD (Ahrens et al., 2016, Barley et al., 2018, Gratton et al., 2015, Sukumaran and Knowles, 2017). This includes processes such as IBD, which we identified in our mainland samples, that can result in oversplitting species even in well-represented, continuously sampled populations. When considered in conjunction with our relatively sparse sampling, particularly in central Amazonia (Fig. 1), it is most likely that SSDA and BFD mis-interpreted this structure as speciation, and thus oversplit the mainland clade. Additionally, for both the mtDNA and RADseq data, eastern and western populations are not reciprocally monophyletic. While reciprocal monophyly at any specific locus is not a prerequisite for species delimitation (Hudson and Coyne, 2002, Palumbi, 2001), rapidly coalescing loci like mtDNA frequently form monophyletic sister species, reflecting their reproductive isolation (Wiens and Penkrot, 2002, Zink and Barrowclough, 2008). Thus, the failure to recover reciprocal monophyly, coupled with high proportions of shared alleles between eastern and western lineages, supports a single-species hypothesis for mainland, i.e. *G. humeralis sensu stricto*.

Our estimates of the divergence time between mainland Amazonia and Trinidad are moderately disparate (mean = 1.89 mya (secondary calibration) and 2.7 mya (ND2 rate)). This is as expected, because gene divergence occurs prior to species divergence (Edwards and Beerli, 2000). Thus, we err on the side of the more-recent species divergence estimate of 1.89 mya (Fig. 2), which then suggests that cladogenesis between *G. ferrugineus* and *G. humeralis* took place in the early- to mid-Pleistocene, coinciding with the published divergences separating sister taxa in other organisms distributed on Trinidad and South America, including: fishes (Jowers et al., 2008), frogs (Camargo et al., 2009), skinks (Hedges and Conn, 2012), and birds (Hunt et al., 2001). Concordance across animal clades is suggestive of a large-scale isolating event between groups of organisms on Trinidad and South America during this time-period due to Pleistocene glacial cycles. However, these divergences are ancient considering recent connections between the Paria peninsula of Venezuela and Trinidad as recently as 10,000 years ago (Comeau, 1991). This transient connector may have also provided *G. ferrugineus* with the means of re-colonizing the mainland in a similar manner to *G. ceciliae*and *G. vittatus* (Supplemental Fig. 1). This possibility presents an interesting testable hypothesis of testing co-divergence of these lineages. Nonetheless, testing this hypothesis using a model-based biogeographic analysis (such as Ree et al., 2005) is currently not possible, as we are still lacking a fully sampled *Gonatodes* phylogeny (Gamble et al., 2008, Schargel et al., 2010, Russell et al., 2015).

We are currently unable to devise definitive tests to differentiate between three competing phylogeographic scenarios: (1) Trinidad and mainland populations were isolated via vicariance during Pleistocene glacial cycles, (2) dispersal to Trinidad via river flotsam (from the Orinoco or other nearby river), or (3) the inverse scenario, dispersal to the mainland from Trinidad. Given the current data, we are unable to ascertain the approximate distribution of the most recent common ancestor to *G. humeralis* and *G. ferrugineus*. As discussed above, western Amazonian populations are nested within eastern populations of *G. humeralis*, excluding the possibility of an Andean origination (Fig. 1). *G. humeralis*possesses significantly greater genetic diversity in eastern Amazonia than *G. ferrugineus*, which suggests a founder effect bottleneck on Trinidad via (1) vicariance or (2) riverine dispersal and discourages (3) the inverse possibility of dispersal from Trinidad to South America (Supplemental Table 2). In many cases, high levels of genetic diversity correlate with a lineage's point-of-origin as genetic diversity accumulates over time in stable populations (Ingman et al., 2000, Kimura, 1983). In addition, most *Gonatodes* species occur in South America, including a member of *G. humeralis sensu lato's* sister group, *G. conncinatus*, suggesting a continental origin, with Caribbean species resulting from subsequent dispersals from the mainland (Supplemental Fig. 1), unlike *Anolis* lizards (Glor et al., 2001). However, several species closely-related to this clade, e.g. *G. ocellatus*, *G. ceciliae*, and *G. antillensis* (*G. conncinatus*' sister species), occur on islands north of South America, including Trinidad and Tobago (Supplemental Fig. 1). Thus, although the data are suggestive, these scenarios can, and should be, explicitly tested when sufficient data are available.

The identification of the recent radiation of *G. humeralis* across Amazonia provides a powerful framework for testing recent biogeographic theories using fine-scale sampling, given specific demographic and phylogeographic predictions (Avila-Pires et al., 2012, Bush and Oliveira, 2006, Haffer, 1997, Prates et al., 2016, Werneck et al., 2012). We found that *G. humeralis* does not diverge from a neutral model, suggesting a relatively constant population size over time. However, it is also known that small sample sizes (mainland N = 20) can confound true deviations from neutrality, although failure to diverge from a neutral model is also a common theme in Amazonian taxa and is not unique to *G. humeralis* (Lessa et al., 2003). This is still somewhat surprising since the divergence between *G. humeralis* in eastern and western Amazonia has occurred so recently (Fig. 2). This shallow time-frame, however, provides the potential for Quaternary divergence hypotheses, namely the refuge (Haffer, 1969) and vanishing refuge (Vanzolini and Williams, 1981) hypotheses, to be tested by employing more fine-scale sampling than was available for this study. Thus, *G. humeralis sensu stricto* provides a model system for elucidating the recent history of Amazonia.

Within eastern Amazonia, our results are largely concordant with the findings of Avila-Pires et al. (2012), using mitochondrial data to infer high genetic diversity in eastern Amazonia (Supplemental Table 3). Along with the lack of genetic diversity in western Amazonia and on Trinidad, our data suggest the most recent common ancestor of *G. humeralis sensu stricto* occurred in eastern Amazonia, with subsequent westward expansion; as source populations typically have higher genetic diversity than their emigrated counterparts (Cann et al., 1987, Ingman et al., 2000). Previous investigations of geographic barriers that have affected *G. humeralis* have focused on riverine barriers (Avila-Pires et al., 2012). Rivers have played an important role in Amazonian biogeography by acting as barriers to gene flow in multiple taxa [Cracraft, 1985, Haffer, 1969, Oliveira et al., 2017, Wallace, 1852]. However, there is little evidence that they have had much impact on the present-day distribution of *G. humeralis*, as our time-calibrated phylogeny suggests that intraspecific divergence within *G. humeralis* took place <2.4 mya (Fig. 2), which is more recent than the establishment of the present-day Amazon river (≥3.6 mya) or the paleo-Tocantins river (≈2.6 mya) (Figueiredo et al., 2009, Latrubesse et al., 2010). Future investigations, with more thorough geographic sampling, may be able to elucidate a role for riverine barriers in relation to migration and gene flow in *G. humeralis*. Furthermore, the adaptation(s) that have led to the unusually broad distribution of *G. humeralis* may be of greater macroevolutionary importance for further investigation. Here, we briefly discuss the current state of knowledge regarding *G. humeralis'* lineage-specific adaptations.

4.1. Gonatodes as a phylogeographic model system

Gonatodes humeralis is distributed over a geographic range considerably larger than that of any of its congeners. Indeed, because most geckos exhibit small ranges, *G. humeralis* may possess one of the largest native ranges of any gecko species (Meiri et al., 2017, Roll et al., 2017). *Gonatodes humeralis* resembles its congeners in many respects, and there are several hypotheses to explain the large distribution of *G. humeralis*. The first involves increased thermal tolerance*,* which could allow *G. humeralis* to disperse across warm, open areas between forest fragments (Vanzolini and Williams, 1981). However, *G. humeralis* maintains the same body temperature as at least two congeners: *G. concinnatus* (Vitt and Zani, 1996); and *G. hasemani* (Vitt et al., 2000), and although it occupies slightly warmer microhabitats than *G. hasemani*, its thermal properties may be explained by differences in body size; as *G. humeralis* is the smallest member of its genus (Avila-Pires, 1995). To test this as a potential explanation for the relative success of *G. humeralis*, body and microhabitat temperatures for additional *Gonatodes* species will be needed (Hertz et al., 1993). Another hypothesis involves the presence of functionally adhesive digits in *G. humeralis*, and *G. ferrugineus*, a unique trait for these taxa (Higham et al., 2017, Russell et al., 2015).

The gain and loss of adhesive toepads in geckos has been hypothesized to represent a key innovation (Higham et al., 2017, Losos, 2011, Russell and Delaugerre, 2017). A key innovation is a behavioral or morphological adaptation that has the capacity to enhance competitive ability, relax adaptive trade-offs, or catalyze the exploitation of a novel resource, which, in turn enhances the number or longevity of a species (Hunter, 1998). Digital adhesion allows geckos to exploit vertical, low-friction surfaces and may have allowed *G. humeralis* to occupy habitats unavailable to its congeners, such as higher strata in the rainforest canopy or locomotion on a wide variety of substrates (Vitt et al., 1997, Russell et al., 2015). Although current genetic and fossil data are lacking to successfully correlate gain and loss of digital adhesion and diversification rates in geckos, it has been demonstrated that: (1) digital adhesion has been gained, and lost, multiple times throughout the evolutionary history of gecko lizards (Gekkota) (Gamble et al., 2012b), (2) under different environmental conditions, selection can favor the presence or absence of adhesive digits (Russell and Delaugerre, 2017), (3) the evolution of functional adhesion requires few morphological changes (Russell et al., 2015), and (4) small morphological changes can have marked impacts on function and the success of a lineage (Burggren, 1992, Higham et al., 2015, Higham et al., 2016, Hunter, 1998, Liem, 1973Russell, 1979, Thomason and Russell, 1986, Webb, 1982). Although, key innovations are generally discussed in the context of adaptive radiations(Farrell, 1998, Stroud and Losos, 2016), it is evident that we witness evolutionary processes as a snapshot in time and, given a strong environmental impetus, a well-adapted (successful) lineage with a broad range may also be a lineage that is primed for subsequent diversification (Endler, 1977, Haffer, 1969). Thus, digital adhesion, which is absent from all other *Gonatodes* species, provides a putative mechanism for *G. humeralis sensu lato*, relative to other members of the genus*,* to have capitalized on available ecological opportunity across Amazonia and on Trinidad (see [Wellborn and Langerhans, 2014] for a scholarly review of ecological opportunity).

5. Conclusion

We propose that *G. humeralis sensu lato* is composed of two species. (1) *G. humeralis sensu stricto* occupies mainland South America and (2) its sister species, *G. ferrugineus*, resides allopatrically on the island of Trinidad. However, we reject the hypothesis that *G. humeralis* is a species-complex made up of multiple species across Amazonia. More specifically, genetic analyses support the hypothesis that *G. humeralis sensu stricto* is a single species throughout its contiguous range across northern South America with substantial population structure (local diversity and IBD). This is extremely atypical for a small, non-volant Neotropical taxon, and this pattern contrasts with that of most Amazonian taxa, as well as other species of *Gonatodes*, which occupy small, disjunctive distributions, and this discrepancy in geographic range invites further investigation. Indeed, unlike many clades consisting of widespread Neotropical taxa, *Gonatodes* harbors both widespread and geographically restricted taxa, providing a powerful system for identifying traits that influence species distributions. Thus, future work should attempt to elucidate the evolutionary adaptations that have influenced the biogeography of *Gonatodes*.

Author contributions

B.J.P. assisted in study design, performed lab work, analyzed data, and wrote the manuscript. G.R.C. conducted fieldwork. T.E.H. assisted in study design. A.P.R. assisted in study design and manuscript preparation. D.P.S. and L.J.V. conducted fieldwork. T.G. conducted fieldwork, assisted in study design, performed lab work, and manuscript preparation. In addition, all authors read and approved the final manuscript.

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Data accessibility

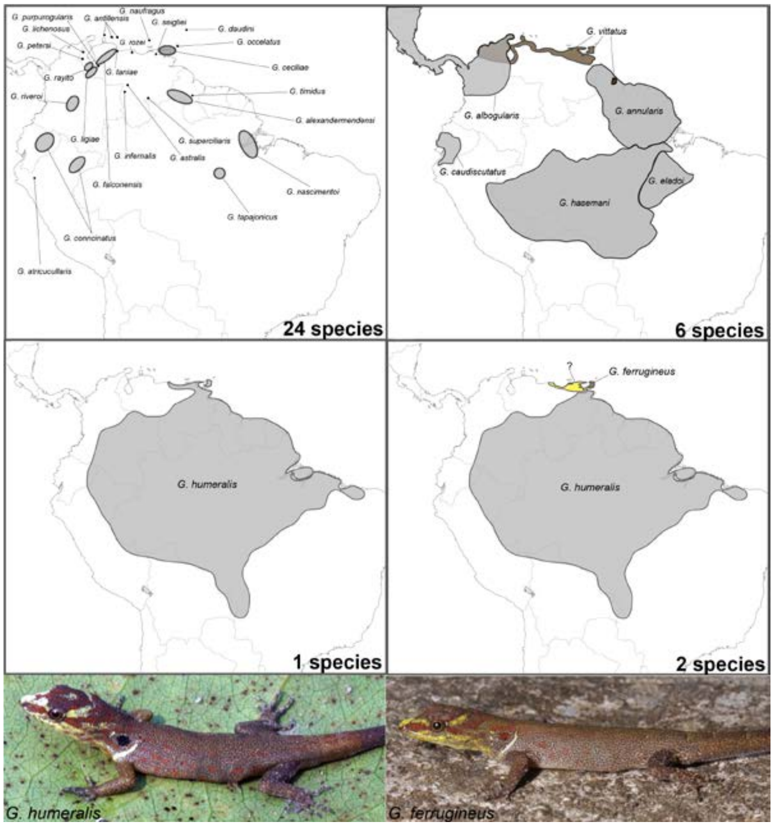
GenBank and Short Read Archive (SRA) accession numbers provided in Supplemental Table 1.

Appendix A. Supplementary material

The following are the Supplementary data to this article:

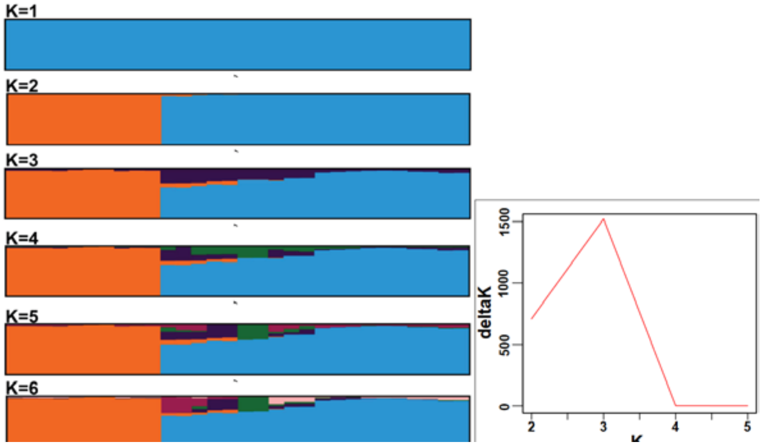
Supplementary data 1.

Supplementary Figure 1. Gonatodes humeralis occurs sympatrically across its range with 13 congeners: G. alexandermendensi, G. annularis, G. ceciliae, G. conncinatus, G. eladoi, G. hasemani, G. nascimento, G. riveroi, G. rozei, G. seigliei, G. tapajonicus, G. timidus, and G. vittatus. All range estimates are based upon museum records from Universidad Nacional de Colombia and from the literature: Avila-Pires, 1995; Carvajal-Campos & Torres-Carvajal, 2012; Meilink et al. 2013; Ribeiro-Junior, 2015; Rivas & Schargel, 2008; Rivero-Blanco, 1979; Schargel et al. 2017; Uetz, 2017; Vanzolini, 1955; Vitt & Zani, 1996; Vitt et al. 1997 & 2000. Bottom right: Proposed species range adjustments concluded from this study, i.e. dividing G. humeralis and G. ferrugineus. "**?**" denotes the findings of Rivero-Blanco (1979) where male specimens from northern Venezuela more closely resemble specimens from Trinidad leading to the possibility that this is G. ferrugineus. Bottom: Representative photographs of each species G. humeralis courtesy of L.J.V. and G. ferrugineus courtesy of D.P.S.



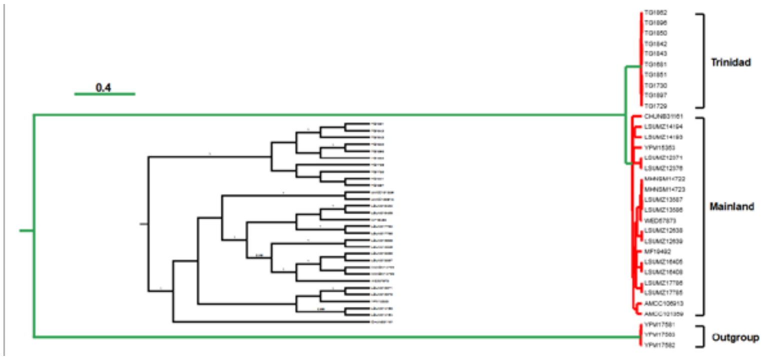
Supplementary data 2.

Supplementary Figure 2. Distruct plot output of STRUCTURE software investigating proportions of shared alleles among 16,381 unlinked SNPs with K=1-6. Line graph depicting deltaK = [mean(|L”(K)|)/sd(L(K))] over iteration of each K.



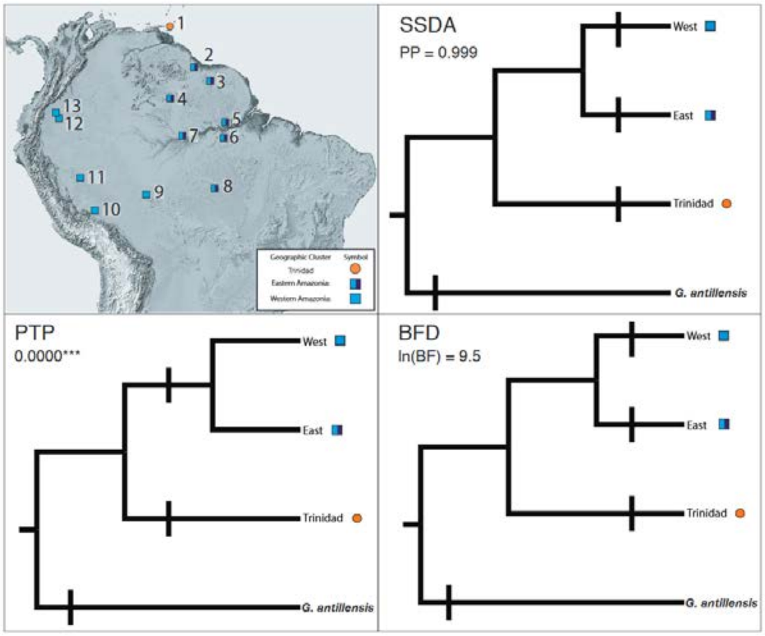
Supplementary data 3.

Supplementary Figure 3. Poisson Tree Processes (PTP) annotated mitochondrial tree output indicating distinct species as red clades with green root. The same tree is shown in an expanded cladogram format reporting nodal support of the mtDNA tree (posterior probabilities ≥0.95).



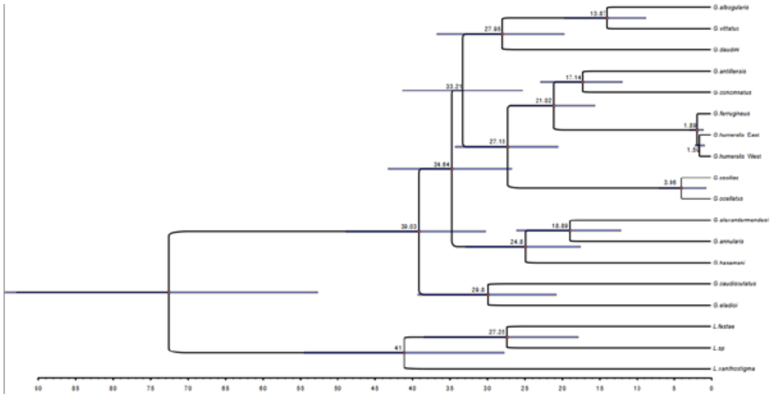
Supplementary data 4.

Supplementary Figure 4. Top left: map relating locality data to location on representative trees. Bottom left: (PTP) Species delimitation model predicted by PTP analysis using mitochondrial gene tree. Top right: (SSDA – STACEY) Species delimitation model predicted by SSDA in a Bayesian coalescent framework, support values for each node correspond to posterior probabilities that indicate each node is a distinct species under six different demographic scenarios (see methods). Bottom right: (BFD) Species delimitation model supported by BFD, reported number [ln(BF) = 9.5] reflecting a model comparison between the two-species (PTP) and three-species (SSDA) models by comparing Bayes factors (see methods). Dash marks along branch lengths represent that the lineage beyond is supported as a species-level clade in that respective analysis.



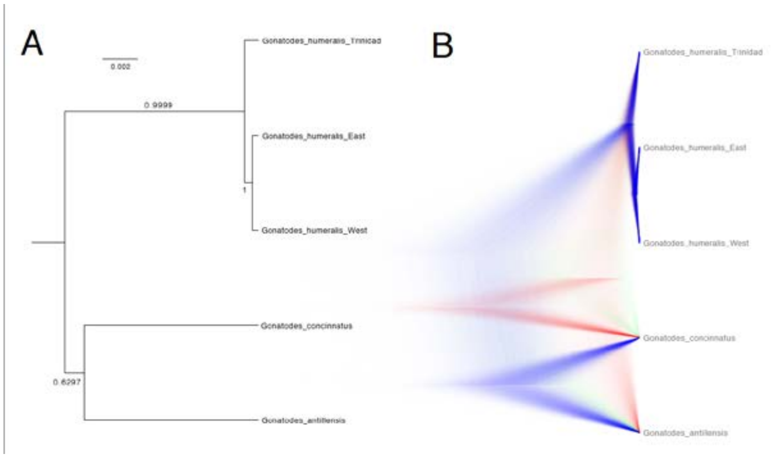
Supplementary data 5.

Supplementary Figure 5. Bayesian time-calibrated phylogenetic reconstruction using a secondary time-calibrated root acquired from Gamble et al. (2015), set at ≈72.5 million years with a normal distribution (σ = 4). Posterior probabilities reported at each node with colored circles where large red circles are equal to posterior probability equal to ≥0.99, while smaller blue circles are equal to less than 0.95 (unsupported). Mean node ages are reported with node bars indicating 95% confidence interval of each mean age.



Supplementary data 6.

Supplementary Figure 6. Bayesian phylogenetic reconstruction using STACEY. (A) Maximum sum of clade credibility tree summarized from 27,003 post-burnin trees, numbers are posterior support for the associated node. (B) DensiTree representation of 27,003 post-burnin trees.



Supplementary data 7.

Supplementary Table 1. Locality and GenBank accessibility information of data generated in this study.

Species	**Specimen ID**	**Locality**	**Locality ID**	**16S**	**ND2**	**RAG 1**	**CMOS**	**PTPN 12**	**MAP 1b**	**Radseq**
G. ferrugineus	TG1681	Biche, Trinidad	1	KX762113	KX762236	KX762207	KX762257	KX762141	KX762166	SAMN06827899
G. ferrugineus	TG1729	Flanagin Town, Trinidad	1	KX762114	KX762237	KX762208	KX762250	KX762142	KX762167	SAMN06827900
G. ferrugineus	TG1730	Flanagin Town, Trinidad	1	KX762115	KX762238	KX762209	KX762255	KX762143	KX762168	SAMN06827901
G. ferrugineus	TG1842	Biche, Trinidad	1	KX762116	KX762239	KX762210	KX757678	KX762144	KX762169	SAMN06827902
G. ferrugineus	TG1843	Biche, Trinidad	1	KX762117	KX762240	KX774276	N/A.	KX774278	KX762170	SAMN06827903

G. ferrugineus	TG1850	Flanagin Town, Trinidad	1	KX762118	KX762241	KX762211	KX762254	KX762145	KX762171	SAMN06827904
G. ferrugineus	TG1851	Flanagin Town, Trinidad	1	KX762119	KX762242	KX762212	KX762253	KX762146	KX762172	SAMN06827905
G. ferrugineus	TG1862	Flanagin Town, Trinidad	1	KX762120	KX762243	KX762213	KX762252	KX762147	KX762173	SAMN06827906
G. ferrugineus	TG1896	Flanagin Town, Trinidad	1	KX762121	KX762244	KX762214	KX762256	KX762148	KX762174	SAMN06827907
G. ferrugineus	TG1897	Flanagin Town, Trinidad	1	KX762122	KX762245	KX774277	KX762251	KX774279	N/A.	SAMN06827908
G. humeralis	AMCC101359	Kwakwani, Berbice River, Guyana	2	EU477057	KX762218	KX762193	KX762179	KX762126	KX762152	SAMN06827879
G. humeralis	AMCC106913	Dubulay Ranch, Berbice River, Guyana	2	EU477055	KX762219	N/A.	N/A.	N/A.	N/A.	SAMN06827880
G. humeralis	YPM15353	Kappel, Sipali District, Suriname	3	EF564012	KX762247	N/A.	N/A.	N/A.	N/A.	SAMN06827898
G. humeralis	LSUMZ12371	Faz. Nova Esperanca, Roraima, Brazil	4	EU477058	KX762222	KX762194	KX762180	KX762127	KX762153	SAMN06827882
G. humeralis	LSUMZ12376	Faz. Nova Esperanca, Roraima, Brazil	4	EU477056	KX762223	N/A.	N/A.	N/A.	N/A.	SAMN06827883
G. humeralis	CHUNB31161	Monte Alegre, Pará, Brazil	5	EF564040	KX762220	N/A.	EF564092	N/A.	N/A.	SAMN06827881
G. humeralis	LSUMZ14193	Santarem, Pará, Brazil	6	EF564031	KX762224	KX762196	EF564085	KX762129	KX762155	SAMN06827888
G. humeralis	LSUMZ14194	Santarem, Pará, Brazil	6	EF564029	KX762225	KX762195	KX762181	KX762128	KX762154	SAMN06827889
G. humeralis	LSUMZ16405	Castanho, Amazonas, Brazil	7	EU477063	KX762230	KX762201	KX762184	KX762134	KX762160	SAMN06827890
G. humeralis	LSUMZ16408	Castanho, Amazonas, Brazil	7	EU477062	KX762231	KX762202	KX762185	KX762135	KX762161	SAMN06827891
G. humeralis	CHUNB47049	Alta Floresta, Mato Grosso, Brazil	8	EU477054	KX762221	N/A.	N/A.	N/A.	N/A.	N/A.

G. humeralis	LSUMZ 17785	Guajará-Mirim, Rondônia, Brazil	9	EU477065	KX762232	KX762203	KX762186	KX762136	KX762162	SAMN06827892
G. humeralis	LSUMZ 17786	Guajará-Mirim, Rondônia, Brazil	9	EU477064	KX762233	KX762204	KX762187	KX762137	KX762163	SAMN06827893
G. humeralis	MHNS M14722	Cuzco Amazònico, Madre de Dios, Peru	10	EU477067	KX762234	KX762205	KX762188	KX762138	KX762164	SAMN06827895
G. humeralis	MHNS M14723	Cuzco Amazònico, Madre de Dios, Peru	10	EU477068	KX762235	KX762206	KX762189	KX762139	KX762165	SAMN06827896
G. humeralis	WED57873	Cuzco Amazònico, Madre de Dios, Peru	10	EF564028	KX762246	KX762215	EF564082	KX762140	KX762175	SAMN06827897
G. humeralis	LSUMZ 13586	Porto Walter, Acre, Brazil	11	EU477069	KX762228	KX762199	KX762183	KX762132	KX762158	SAMN06827886
G. humeralis	LSUMZ 13587	Porto Walter, Acre, Brazil	11	EU477066	KX762229	KX762200	N/A.	KX762133	KX762159	SAMN06827887
G. humeralis	MF19492	Orellana, Napo, Ecuador	12	EF564013	JX041361	EF534796	EF534922	JF416860	N/A.	SAMN06827894
G. humeralis	LSUMZ 12638	RPF-Cuyabeno, Sucumbíos, Ecuador	13	EU477061	KX762226	KX762197	KX762182	KX762130	KX762156	SAMN06827884
G. humeralis	LSUMZ 12639	RPF-Cuyabeno, Sucumbíos, Ecuador	13	EF564030	KX762227	KX762198	EF564084	KX762131	KX762157	SAMN06827885
G. antillensis	YPM17581	Westpunt Bay Beach, Curaçao	N/A.	KX762123	KX762248	KX762216	KX762191	KX762149	KX762176	N/A.
G. antillensis	YPM17582	Westpunt Bay Beach, Curaçao	N/A.	KX762124	KX762249	KX762217	KX762192	KX762150	KX762177	N/A.
G. antillensis	YPM17583	Westpunt Bay Beach, Curaçao	N/A.	KX762125	KP640636	KP640630	KP640623	KX762151	KX762178	N/A.

Supplementary data 8.

Supplementary Table 2. GenBank accessibility for StarBEAST2 analysis from Higham et al. (2017) and this study

Species	**16S**	**ND2**	**ACM4**	**RAG1**	**RAG2**	**CMOS**	**PTPN12**	**PDC**
G. ferrugineus	KX762113	KX762236	--	KX762207	--	KX762257	KX762141	--
G. ferrugineus	KX762119	KX762242	--	KX762212	--	KX762253	KX762146	--
G. ferrugineus	KX762120	KX762243	--	KX762213	--	KX762252	KX762147	--

*G. humeralis*_East	EU477057	KX762218	EF564057	KX762193	EF564109	KX762179	KX762126	--
*G. humeralis*_East	EF564040	KX762220	EF564066	--	EF564118	EF564092	--	--
*G. humeralis*_East	EF564031	KX762224	EF564059	KX762196	EF564111	EF564085	KX762129	--
*G. humeralis*_West	EF564028	KX762246	EF564056	KX762215	EF564108	EF564082	KX762140	--
*G. humeralis*_West	EF564013	JX041361	EF534879	EF534796	EF534964	EF534922	JF416860	--
G. antillensis	KX762123	KX762247	--	KX762216	--	KX762191	KX762149	--
G. antillensis	KX762124	KX762248	--	KX762217	--	KX762192	KX762150	--
G. antillensis	KX762125	KP640636	KP640627	KP640630	KP640625	KP640623	KX762151	--
G. eladioi	HQ426195	--	EF564055	HQ426283	EF564107	EF564081	JF416859	HQ426195
G. hasemani	EF564015	KT119569	EF534878	--	EF534963	EF534921	--	EF534837
G. albogularis	EF564020	JX041354	EF534880	EF534797	EF534965	EF534923	JF416854	EF534839
G. alexandermendesi	EF564026	JX041355	EF534881	EF534798	EF534966	EF534924	--	EF534840
G. annularis	EF564037	JX041356	EF534876	EF534794	EF534961	EF534919	JF416855	EF534835
G. caudiscutatus	EF564011	JX041357	EF534877	EF534795	EF534962	EF534920	JF416856	EF534836
G. ceciliae	EF564035	JX041358	EF564062	JF416914	EF564114	EF564088	JF416857	HQ426193
G. concinnatus	EF564012	JX041359	EF564044	HQ426282	EF564096	EF564070	JF416858	HQ426194
G. daudini	EF564034	JX041360	EF534875	EF534793	EF534960	EF534918	--	EF534834
G. ocellatus	EF564014	JX041362	EF564046	HQ426284	EF564098	EF564072	--	HQ426196
G. vittatus	EF564032	JX041363	EF564060	HQ426285	EF564112	EF564086	--	KT119568
L. festae	EF564007	--	EF564042	HQ426297	EF564094	EF564068	JF416862	HQ426208
L. sp.	EF564008	JX041375	EF534871	EF534789	EF534956	EF534914	JF416861	EF534830
L. xanthostigma	EF564009	JX041376	EF534872	EF534790	EF534957	EF534915	JF416863	EF534831

Supplementary data 9.

Supplementary Table 3. Within-group measures of genetic diversity using mtDNA. Within-group genetic distances for each cluster (p-distances) with standard error (S.E.) calculated using 500 bootstrap replicates in

MEGA7 (Kumar et al. 2016). Nucleotide diversity (π) for each cluster and standard deviations (sd), calculated using DNAsp (Librado & Rozas, 2009); including 99% confidence intervals (π±3*sd).

Geographic Cluster	**within-group p-distance**	**S.E.**	**pi**	**sd**	**pi (±) 3*sd *****
G. humeralis East	0.0578	±0.0045	0.05811	0.0032	0.04851 - 0.06771
G. humeralis West	0.0296	±0.0033	0.02761	0.00514	0.01219 - 0.04303
G. humeralis Trinidad	0.0026	±0.0012	0.00284	0.00032	0.00188 - 0.0038
G. antillensis	0.0074	±0.0023	0.0071	0.00206	0.00092 - 0.01328
	MEGA7 (Kumar et al. 2016)		**DNAsp v5.0 (Librado & Rozas, 2009)**		

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