Mitochondrial genome analyses uncover intriguing population structure across the invasive trajectory of an iconic invader

Running title: Intriguing population structure of an invader

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Abstract

Invasive species offer insights into rapid adaptations in novel environments. The iconic cane toad (*Rhinella marina*) is an excellent model for studying rapid adaptation during invasion. Previous research using the mitochondrial NADH dehydrogenase 3 (*ND3*) gene in the Hawai'ian and Australian invasive populations found a single haplotype, indicating an extreme genetic bottleneck following introduction. Nuclear genetic diversity also exhibited reductions across the genome in these two populations. Here, we investigated the mitochondrial genome diversity of cane toads across this invasion trajectory. We created the first reference mitochondrial genome of the cane toad with long-read sequencing and constructed a phylogeny of Anura full mitochondrial genomes. We used transcriptomic data of 125 individuals from the native (French Guiana) and introduced (Hawai'i and Australia) ranges to construct nearly-complete mitochondrial genomes for population genomics analyses. As expected, the cane toad belongs to family Bufonidae, distinct from genus *Bufo*. In agreement with previous investigations of these populations, we identified genetic bottlenecks in both Hawai'ian and Australian introduced populations, alongside evidence of population expansion in the invasive ranges. Although mitochondrial genetic diversity in introduced populations was reduced, our results revealed that it had been underestimated: we identified 45 mitochondrial haplotypes in Hawai'ian and Australian samples, none of which were found in the native range. Additionally, we identified two distinct groups of haplotypes from the native range, separated by a minimum of 110 base pairs (0.6%). These findings enhance our understanding of Anura phylogenetics and how invasion has shaped the genetic landscape of this species.

Keywords

Rhinella marina, population genomics, mitochondrial genome, haplotypes, invasion biology

Introduction

Invasive species are a substantial global concern, particularly due to likely range expansions under climate change (Bonebrake et al., 2018; Seebens et al., 2017), an increasing rate of anthropogenic introductions (Lowe, Browne, Boudjelas, & De Poorter, 2000), and their negative impacts on native biodiversity and the economy (Bradshaw et al., 2016; Mainka & Howard, 2010). Invasive species often rapidly adapt to their new environments (Rollins et al., 2013), creating an opportunity to study the process of rapid evolution. Biological invasions sometimes present a genetic paradox where, despite small founding population sizes that often reduce genetic diversity in the introduced range (i.e. genetic bottlenecks), they still maintain the ability to readily adapt to novel conditions (Allendorf & Lundquist, 2003; Estoup et al., 2016; Schrieber & Lachmuth, 2017).

Various genetic markers, including those from the nuclear and mitochondrial genomes, have been employed to investigate the introduction history and evolutionary processes of invasive species. Because of their distinct mode of inheritance and mutation rates, nuclear and mitochondrial markers may yield different insights into the evolutionary events underlying invasion (Toews & Brelsford, 2012). Genetic variation in invasive species is shaped by complex interplays among genetic drift, demography and selection. Small founding populations, common during an invasion, can promote genetic drift, leading to reduced genetic diversity. Furthermore, whilst genetic drift results in less efficient selection (Gravel, 2016), populations expanding their range into new environments may experience novel selection regimes (Sakai et al., 2001) and spatial sorting (Shine, Brown, & Phillips, 2011) that further reduce genetic diversity.

Mitochondrial DNA (mtDNA) is more susceptible to genetic drift than nuclear markers due to its uniparental inheritance and small effective population size (Klucnika & Ma, 2019; Xiao, Nguyen, Wu, & Hao, 2017). Nonetheless, mtDNA has been extensively used to study the evolutionary history of different species or populations within the same species because of its non-recombining nature and purported near-neutrality. However, the neutrality of mtDNA has been challenged by evidence of selection, likely due to the important role of mitochondria in ATP production and cellular activities (Meiklejohn, Montooth, & Rand, 2007). Deleterious mutations are rapidly removed by purifying selection to maintain the function of the electron transport chain. Further, variation in mtDNA has been linked to adaptation to high latitudes (Chen et al., 2020; Wang et al., 2021) and temperature (Baker et al., 2019; Cheng, Liu, Yang, Sun, & Kong, 2013; Silva, Lima, Martel, & Castilho, 2014). There exists a substantial repository of mtDNA data in public databases, enabling comprehensive comparative genetic analyses, increasing its usefulness as a marker.

The cane toad (*Rhinella marina*) is one of the world's most infamous, and well-studied, invasive species (Lowe et al., 2000). Cane toads are native to Central and South America (Zug & Zug, 1979) and have been introduced to multiple Caribbean islands for biological control of cane beetles, a parasite of sugarcane (Easteal, 1981). Cane toads were transported from the Caribbean to Hawai'i in 1932 for cane beetle control and, in 1935, 101 Hawai'ian cane toads were translocated from Oahu to Queensland, Australia (Turvey, 2013). However, they failed to control cane beetles in Australia and, facilitated by a high reproductive rate, subsequently spread across the continent to arid Western Australia. This species' inexorable spread throughout its introduced range in Australia has had catastrophic

effects on the naïve predators it has encountered (Chinchio et al., 2020). Freshwater crocodiles, snakes, lizards, and quolls have been particularly badly affected by the cane toad invasion in Australia (Shine, 2010). The colonization of the cane toad is still on-going; toads occupy over 1.2 million km^2 of Australia (Urban, Phillips, Skelly, & Shine, 2008) including tropical Queensland, the Northern Territory, and the Kimberley region of northern Western Australia (Radford et al., 2019).

Like in many other invasions, the cane toads' translocation resulted in genetic bottlenecks. Australian cane toads have very low MHC class I and class II (Lillie, Cui, Shine, & Belov, 2016; Lillie, Dubey, Shine, & Belov, 2017; Lillie, Shine, & Belov, 2014) and microsatellite (Leblois, Rousset, Tikel, Moritz, & Estoup, 2000) diversity. Previous analysis of the NADH dehydrogenase 3 (*ND3*) mitochondrial gene from 31 individuals sampled from Hawai'i and Australia identified a single haplotype, a substantial reduction of haplotype diversity compared to the native range (Slade & Moritz, 1998), further supporting the presence of genetic bottlenecks in the invasive ranges. Despite this low genetic variation, phenotypic traits differ substantially across the cane toad's Australian range including immune function, dispersal ability, and behaviour (Alford, Brown, Schwarzkopf, Phillips, & Shine, 2009; G. P. Brown, Phillips, Dubey, & Shine, 2015; Gruber, Brown, Whiting, & Shine, 2018; Gruber, Whiting, Brown, & Shine, 2017; Phillips, Brown, & Shine, 2010). Some of these traits have been shown to be intergenerationally transmitted, consistent with genetic causes. For example, toads from range-core and range-edge populations that were raised in a common-garden experiment showed divergence of behavioural traits and morphology (Gruber, Brown, Whiting, & Shine, 2017; Hudson, Brown, & Shine, 2016). The invasion success of this

species in Australia, coupled with evidence of evolutionary change since introduction make it an ideal system for the study of rapid evolution during invasion.

Despite extensive research into toad evolution in the introduced range, we need improved genetic resources to clarify the molecular mechanisms underlying these changes. Transcriptome data from spleen, muscle and/or brain tissues of toads from French Guiana, Hawai'i, and Australia, and liver tissue from Brazil exist (Richardson et al., 2018; Rollins, Richardson, & Shine, 2015; Selechnik, Richardson, Shine, Brown, & Rollins, 2019; Yagound et al., 2022) and a draft reference genome (Edwards et al., 2018) is available, but population genomic analyses of cane toads across this invasion trajectory have thus far been restricted to a single study of single nucleotide polymorphisms (SNPs) from the nuclear genome (Selechnik, Richardson, Shine, DeVore, et al., 2019). Here, we present a complete cane toad mitochondrial genome (mitogenome) and explore the species' phylogenetic relationships with other Anurans. We then investigate evolution across the invasion trajectory using mtDNA data derived from RNASeq and whole-genome resequencing. We compare our data to those of a previously-published study of *ND3* on the same populations (Slade & Moritz, 1998), to determine whether our whole-mitochondrial genome analysis can extend population-genetic inferences. We predicted that there would be decreasing genetic diversity within the mitochondrial genome with increasing distance from the native range due to genetic bottlenecks. We also predicted that we would identify more genetic diversity within Australia than has been previously characterised, given the phenotypic evidence of adaptation to this novel environment.

131 # Materials and Methods

132 ## Reference mitochondrial genome

133 High-molecular-weight genomic DNA was extracted from the liver of a female cane toad
134 collected in the Kimberley region of Western Australia and sequenced on Illumina HiSeq X
135 Ten and PacBio RS II platforms as described previously (Edwards et al., 2018). The
136 mitogenome was assembled from 36 SMRT cells generated from the first two SMRTBell
137 libraries (ENA experiments ERX2389178 and ERX2389179).

138 The 17,757 basepair (bp) *Bufo japonicus* complete mitochondrial genome (gi:157939553)
139 was downloaded from NCBI on 29/3/2016 and used to identify mitochondrial subreads from
140 the raw sequencing data. GABLAM (Davey, Shields, & Edwards, 2006) was used to map the
141 *B. japonicus* mtDNA onto PacBio subreads using blastn (blast+ v2.2.31) and extract 143
142 subreads that had 75%+ bidirectional coverage, *e.g.* at least 75% of the subread mapped
143 onto the mtDNA and at least 75% of the mtDNA was covered by the subread. Any subreads
144 that mapped the negative strand were reverse complemented. The subread with the best
145 global hit to the *B. japonicus* mtDNA was selected as the core of the cane toad
146 mitochondrial genome. A second GABLAM search was performed of the 143 subreads
147 against this new query, retaining 29 sequences that had >80% global sequence identity
148 within local matches in the same orientation and order, excluding any “wrapping” due to
149 circularity. These 29 sequences were aligned using MAFFT v2.273 (Katoh & Standley, 2013)
150 with a very small gap penalty to account for the high indel rate (--localpair --maxiterate 1000
151 --op 0.1 --ep 0.1 --lop -0.1 --nuc). SeqSuite (Edwards, Paulsen, Aguilar Gomez, & Perez-
152 Bercoff, 2020) was used to generate a consensus sequence from the alignment using the
153 most frequent non-gap base call at each position. Any column with fewer than 5 non-gap

154 sequences was excluded from the consensus. All 143 raw subreads were mapped onto the
155 consensus using BLASR and the sequence was polished using Quiver (SMRT Analysis v2.3.0).
156 GABLAM was used to identify the overlapping ends of the sequence, which was then
157 circularised by trimming off the first 1957 bp for a final length of 18,152 bp. A final polishing
158 step was performed using the Illumina data (ENA experiment ERX2845325), mapped with
159 Bowtie v2.3.3 (Langmead & Salzberg, 2012) and error-corrected with Pilon v1.20 (Walker et
160 al., 2014).

161 The mitochondrial genome was annotated using MITOS online (Bernt et al., 2013) with the
162 vertebrate mtDNA genetic code (Genetic Code Table 02). Annotated protein-coding genes
163 were manually curated and extended to include stop codons where required. Where no
164 canonical in-frame stop codon was found, it was assumed that a partial (T or TA) stop codon
165 would be completed upon polyadenylation. Codon usage was calculated using
166 https://www.bioinformatics.org/sms2/codon_usage.html (Accessed: 2023-03-29). The final
167 annotated mitochondrial genome was uploaded to NCBI (Accession number: NC_066225.1).
168 The mitogenome map was drawn using the CGView server
169 (http://stothard.afns.ualberta.ca/cgview_server/).

170 ## Phylogenetic analysis

171 Complete mitochondrial genomes of 203 Anura, and 5 non-Anura outgroup species from
172 Caeciliidae and Caudata), were downloaded from NCBI BioProject database (Table S3,
173 Downloaded 2022-09-11). Each mitochondrial genome was re-circularised and edited to end
174 with the control region for subsequent analyses. The multiple sequence alignment was
175 conducted with the cane toad reference mitogenome using MAFFT with auto mode to find

176 the best alignment algorithm (Katoh & Standley, 2013). The resulting alignment was visually
177 inspected using AliView (Larsson, 2014) without further trimming. Maximum likelihood
178 phylogenetic analysis was conducted using IQ-Tree multicore version 2.2.2.7 (default
179 parameters, ultrafast bootstrap (UFBoot) and SH-aLRT branch test with 10000 replicates)
180 (Minh et al., 2020). Clades are considered reliable if SH-aLRT value >= 80% and UFboot value
181 >=95%. The best fit model was found by ModelFinder embedded in IQ-Tree
182 (Kalyaanamoorthy, Minh, Wong, von Haeseler, & Jermiin, 2017). The phylogenetic tree was
183 visualised and edited using R package *ggtree* (Yu, Smith, Zhu, Guan, & Lam, 2017).

184 Sequence data for population- and species-level analyses

185 Previously published cane toad RNASeq data from different tissues were retrieved and
186 downloaded from NCBI BioProject PRJNA510261 and PRJNA395127 (spleen), PRJNA479937
187 (brain) and PRJNA277985 (muscle) for analysis. A total of 125 individuals with 144 tissue
188 samples were included, sampled in French Guiana (*n* = 24), Hawai'i (*n* = 8) and Australia (*n* =
189 93) (Figure 1; Table S1). For some individuals, RNASeq data from both spleen and brain were
190 available; in such cases, we used data from brains. Raw reads were processed as described
191 previously with STAR v2.7.2b (Dobin et al., 2013) and Genome Analysis Toolkit (GATK)
192 v4.1.9.0 (McKenna et al., 2010), using the extracted mitogenome from the draft genome as
193 a reference (Selechnik, Richardson, Shine, DeVore, et al., 2019).

194 Genomic DNA was extracted from fifteen cane toad samples, including two from toe clips,
195 two from spleen, four from liver and seven from muscle tissues using a Gentra PureGene
196 DNA extraction kit (QIAGEN) (Table S1).

197 For two samples (RMH006 and RM265), paired-end sequencing libraries were prepared
198 using the TruSeq DNA PCR-free kit (Illumina). These libraries were sequenced on the
199 Illumina NovaSeq 6000 platform at the Ramaciotti Centre for Genomics, University of New
200 South Wales, Sydney, Australia (UNSW Sydney). The remaining thirteen samples had paired-
201 end sequencing libraries constructed according to the manufacturer's specifications for the
202 NEBNext Ultra DNA Library Prep Kit for Illumina (NEB). Three samples (RMF031, RMF042
203 and RMF044) were sequenced on the 10X Genomics Chromium platform at the Ramaciotti
204 Centre for Genomics, UNSW Sydney. The remaining libraries were sequenced on the
205 Illumina NovaSeq 6000 platform at Deakin Genomic Centre, Victoria, Australia with a target
206 depth of 20-fold coverage per genome. Raw sequencing reads from each sample underwent
207 initial processing to remove adapter sequences. Trimmomatic v0.38 (Bolger, Lohse, &
208 Usadel, 2014) was employed using the following parameters: ILLUMINACLIP:TruSeq3-
209 PE.fa:2:30:10:4 SLIDINGWINDOW:5:30 AVGQUAL:30 MINLEN:36.

210 Mitochondrial segments, *ND3* gene with tRNA-Gly and tRNA-Arg flanking sequence (*ND3*
211 dataset hereafter) were obtained from previous studies (Acevedo, Lampo, & Cipriani, 2016;
212 Slade & Moritz, 1998) (Table S1). We aligned our data with this *ND3* dataset using the R
213 package DECIPHER (Wright, 2016) and trimmed our data to match the existing sequences.

214 Construction of mtDNA genome and variant calling

215 GATK HaplotypeCaller (Poplin et al., 2018) was used to call SNPs and indels using the
216 following criteria: soft clipped bases were not included and variants with minimum Phred-
217 scaled confidence of 20 threshold were kept. To filter low quality variants, GATK
218 VariantFiltration (Van der Auwera, 2020) was used to remove variants in the resulting VCF

219 file: clusters of 3 SNPs within a window of 35 bases, variants with Fisher Strand (FS) greater
220 than 30.0, QualbyDepth (QD) less than 2.0, depth of coverage (DP) less than 20.0 and allelic
221 frequency (AF) less than 0.05. Due to the difficulty of mapping short-read RNASeq data to
222 sequences with multiple repeats, and the risk of introducing false-positive variant calls, we
223 excluded variants from the repetitive sequences in the hypervariable region 2 (HV2) of the
224 mitochondrial control region. We determined the consensus sequence of each sample using
225 FastaAlternateReferenceMaker (McKenna et al., 2010).

226 Consensus mitogenome sequences from individuals with both RNASeq and WGS data were
227 aligned against each other using DECIPHER R packages (Wright, 2016, 2020) using default
228 settings. The alignments were further visualised and analysed with AliView (Larsson, 2014).

229 NUMT identification

230 The presence of NUMTs in the cane toad's genome has not been documented previously.
231 The cane toad draft genome was retrieved and downloaded from NCBI BioProject
232 PRJEB24695. We used RepeatModeler (http://www.repeatmasker.org/RepeatModeler/) to
233 construct species-specific repeat library and masked the genome using RepeatMasker
234 (Tarailo-Graovac & Chen, 2009). NUMTFinder v0.5.3 (Edwards et al., 2021) was used to
235 identify potential NUMTs by searching the full and repeat-masked versions of the nuclear
236 genome with our new mtDNA assembly.

237 Population genetics analyses

238 The spatial distribution of haplotypes was examined by creating a median-joining haplotype
239 network using Network and postprocessed using the maximum parsimony calculation to
240 remove unnecessary median vectors and links (Polzin & Vahdati Daneshmand, 2003). The

241 final network was drawn using Network Publisher v2.1.2.5 (Fluxus Engineering, Clare, UK).
242 This same procedure also was used to analyse the *ND3* dataset.

243 We then investigated population genetic diversity and differentiation. The following
244 diversity indices were calculated using DnaSP v6 (Rozas et al., 2017): nucleotide diversity (π),
245 number of polymorphic sites, number of parsimony informative sites, number of haplotypes
246 (*H*) and haplotype diversity (*h*). Due to the uneven sample size across populations,
247 haplotype richness (*HR*) was calculated using FSTAT v2.9.4 (Goudet, 1995) to measure the
248 number of alleles independent of the sample size. DnaSP was also used to generate a
249 haplotype list for Arlequin software v3.5.2.2 (Excoffier & Lischer, 2010) and a Roehl data file
250 for use with Network software 10.2.0.0 (Bandelt, Forster, & Rohl, 1999; Bandelt, Forster,
251 Sykes, & Richards, 1995). To study the population structure, Arlequin was used to calculate
252 genetic differentiation among and between populations by pairwise fixation indices (*Fst*)
253 and Analysis of Molecular Variance (AMOVA) to estimate the genetic variation among and
254 within populations.

255 To study the demographic history of each population, we used both neutrality statistics and
256 mismatch distribution tests to infer population expansion. Fu's *Fs* statistics was produced
257 using Arlequin and Ramos-Onsins and Rozas R_2 statistics were produced using DnaSP. Fu's *Fs*
258 test calculates the probability of observing a number of haplotype similar or smaller than
259 the observed number of samples (Fu, 1997). Both tests are based on the infinite-site model
260 without recombination, which is suitable for analysing mitochondrial genomic data. A
261 significant negative value of *Fs* indicates an excess of rare alleles and haplotypes due to a
262 sudden population growth after a population bottleneck or genetic hitchhiking while

263 positive values indicate a lack of rare mutations, resulting from balancing selection or
264 population stability. Ramos-Onsins and Rozas R_2 statistic compares the difference between
265 the number of singleton mutations and the average number of segregating sites (Ramos-
266 Onsins & Rozas, 2002). The R_2 statistic is known to have better power than Fu's *Fs* when the
267 sample size is small (< 10) or the number of segregating sites is low (Ramirez-Soriano,
268 Ramos-Onsins, Rozas, Calafell, & Navarro, 2008). A small positive value of R_2 indicates
269 population expansion. The statistical power of these tests was validated using 10,000
270 simulations. Mismatch distributions of the frequency of pairwise differences between
271 haplotype pairs in each population were also examined using the sudden expansion and
272 spatial expansion models implemented in Arlequin (Excoffier, 2004; Schneider & Excoffier,
273 1999). The sum of square deviations (SSD) was used to test the differences between
274 observed and expected values while the raggedness index was used to explore the
275 smoothness of the observed mismatch distribution. The statistical significance of these tests
276 was validated with 500 replicates in Arlequin. A non-significant SSD value and a small
277 raggedness index value with a unimodal distribution indicates no deviation of the observed
278 data from the expectation under the expansion model.

279 Results

280 Summary of the cane toad reference mitochondrial genome

281 The mitogenome of *R. marina* (Figure 2) consists of 18,152 bp, including 22 tRNA genes, 2
282 rRNA genes and 13 protein-coding genes, with 28 genes located on the forward (+) strand
283 and 9 located on reverse (-) strand (Table S2). There is also a 2,753 bp GC-rich control region
284 containing the origin of replication. Two groups of tandem repeats were observed in the
285 latter part of the control region: 530 bps with 6-full repeats and 350 bps with 3-full repeats.

286 The gene order of the cane toad mitogenome was examined by aligning with the members
287 of its sister clades (*Bufo* and *Bufotes*) and we found no gene rearrangements. The base
288 composition of the mitogenome was A: 29%, T: 33%, G: 14% and C: 24%, with a bias with
289 62% of A + T. The codon usages of those with nucleotide G in the third codon position were
290 lower than those with A and T. GCG (Ala) was the least used codon and TGG was the only
291 codon used for encoding Tryptophan. All protein-coding genes used ATG as start codon.

292 Phylogenetics analysis

293 We produced a maximum likelihood phylogenetic tree using whole mitogenomes of Anura.
294 The best fit model was GTR+F+R9 according to lowest Bayesian information Criterion (BIC).
295 The location of *R. marina* in the phylogeny of Anura is shown in Figure 3. *Rhinella marina*
296 was grouped with *Anaxyrus americanus*, an American Bufonid and previously classified
297 under *Bufo* genus, with 100% statistical support in both UFBoot and SH-aLRT in branch tests.
298 Four species of *Bufotes* and four of *Bufo* were well resolved into two subclades. Even though
299 there was high branch support for differentiation between the genera *Rhinella* and
300 *Anaxyrus*, and high support for species within *Bufotes* and *Bufo*, the divergence among
301 genera was not significantly supported, which may indicate the signals in the whole
302 mitochondrial genomes were insufficient for resolving these clades or that wider taxonomic
303 coverage is required to resolve this phylogeny.

304 Verification of mtDNA genomes from RNASeq data

305 A near-complete mitochondrial genome with 17,261 to 17,270 bps (95.1% of reference
306 mitochondrial genome) for a total of 132 individuals was generated from the RNASeq and
307 WGS dataset. A sequence with length of 889 bases consisting of repeats in the HV2 section
308 of the control region was excluded due to difficulty in correctly mapping sequencing reads

309 on repeat regions. To determine the accuracy of our RNASeq-derived mtDNA sequences, we
310 then compared the sequences of the six individuals for which we had both RNASeq and
311 WGS data (Table S4). We identified four sites (site 2244, site, 5162, site 7162, site 7920) that
312 showed intra-individual variation across the two datasets in multiple individuals (i.e.
313 potential RNA editing). Two sites in coding regions (site 267, site 477) had intra-individual
314 differences in only one individual. Additionally, one individual had 12 sites in the control
315 region that differed across datasets (Table S4). We replaced the sites with intra-individual
316 variation in multiple individuals with Ns, minimizing variation for subsequent population
317 analyses that might be introduced by RNA editing.

318 ## NUMT analysis

319 We found 42 mtDNA fragments (38 – 140 bps in length) that mapped to 42 individual
320 contigs of the cane toad draft nuclear genome with over 80% identity (Table S5). All
321 fragments corresponded to the control region of the mitogenome. After the draft genome
322 was masked for repeat elements, no mtDNA fragments were found to map to the nuclear
323 genome.

324 ## Population Genetic Analyses

325 We identified 262 (1.30%) polymorphic sites in the RNASeq dataset, 198 of which were
326 parsimony-informative, including 25 from RNA genes, 145 from protein-coding genes, and
327 28 from non-coding regions and the control region (Table S2), which resulted in the
328 identification of 58 haplotypes (Table 1; Figure 4). The thirteen native samples contained
329 twelve unique haplotypes. Two haplotypes found in all introduced populations accounted
330 for nearly 50% of the sampled individuals. Within introduced populations, a “complex-star”

331 shape topology with mostly singleton mutants was observed and 41 of the 46 introduced
332 haplotypes (89%) were private to a single introduction (Hawai'i: 10; Australia: 31).

333 We also compared the nearly-complete mitochondrial genome dataset with the *ND3*
334 dataset to demonstrate the consistency of haplotype inference across studies, and to put
335 our data into the context of previous work. A total of 24 haplotypes were identified in this
336 analysis (Figure 5); six haplotypes were identified in French Guiana/Guyana, one haplotype
337 was identified in Indonesian samples, three in Hawai'ian and Australian samples, and the
338 remaining haplotypes were from other South America localities. Haplotypes from either side
339 of Andes clearly formed distinct lineages which is consistent to previous work (Acevedo et
340 al., 2016). The single Australian haplotype (Hap1) previously identified in (Slade & Moritz,
341 1998) was the most common haplotype in our Australian samples and the other two
342 haplotypes (Hap2 and Hap3) within Australia were a single nucleotide different to this
343 common haplotype.

344 Haplotype diversity was highest in the native range, with reduced diversity in both
345 Hawai'ian and Australian populations (*h*: 0.9782 vs 0.8800 and 0.8344, respectively) (Table
346 1, Figure 6A). Native toads had the highest nucleotide diversity (π: 0.00469) compared to
347 considerably lower diversity in Hawai'ian and Australian toads (π: 0.00010 and 0.00009,
348 respectively) (Table 1, Figure 6B). Queensland toads had similar haplotype diversity but
349 higher nucleotide diversity and haplotype richness to Hawai'ian toads. The population from
350 the Northern Territory had noticeably lower genetic diversity and haplotype richness than
351 other Australian populations (Table 1, Figure 6A, C). When the relationship between
352 haplotypes across the invasion trajectory is considered, the twelve haplotypes from French

353 Guiana were highly dissimilar to those from the Australian and Hawai'ian populations,
354 forming two distinct clades (Figure 4). These were separated from each other by a minimum
355 of 110 base pair changes and from the common invasive haplotype by a minimum of 38
356 base pair changes (Figure 4). Australian and Hawai'ian haplotypes were intermixed and most
357 of the haplotypes were one base pair different to other haplotypes, with invasive
358 haplotypes being separated from at least one other haplotype by no more than three base
359 pair changes.

360 AMOVA results showed that the overall genetic variation among populations (64.49%) was
361 larger than the variation within populations (36.33%) (Table 2). Pairwise *Fst* values were
362 significant between the native population and all introduced populations (*Fst*: 0.55 – 0.67;
363 Table 3). The *Fst* values between introduced populations were all less than 0.1, indicating
364 substantially less differentiation in the invasive range than in the native range. The
365 Hawai'ian population was significantly differentiated from Australian populations (*Fst*: 0.02,
366 $p = 0.02$), but this difference was driven by the Northern Territory (*Fst*: 0.05, $p = 0.01$ and
367 Western Australian populations (*Fst*: 0.03, $p = 0.01$); Queensland was not significantly
368 differentiated to the Hawai'i population (*Fst*: 0.01, $p = 0.06$). Within Australia, the
369 Queensland population showed significant differentiation to both the Northern Territory
370 and Western Australian populations (*Fst*: 0.04, $p = 0.01$ and 0.03, $p = 0.003$, respectively),
371 whereas the Northern Territory and Western Australian populations were not genetically
372 differentiated (*Fst*: -0.01, $p = 0.72$).

373 Two neutrality tests were implemented to study the demographic history of cane toad
374 populations: Fu's *Fs* and Ramos-Onsins and Rozas' R_2 statistic (Table 4). The native

375 population had non-significant positive Fu's *Fs* values and a large R_2 statistic, indicating the
376 population is not expanding or shrinking (i.e. is demographically stable). Fu's *Fs* values were
377 negative in all introduced populations, indicating an excess of rare haplotypes compared to
378 the expectation from neutrality and all these were statistically significant. R_2 statistics were
379 significant in Hawai'i, Queensland and Western Australia's populations.

380 Mismatch distribution graphs of all populations are shown in Figure S1. The native
381 population showed a multimodal distribution with non-significant SSD and raggedness
382 values, indicating support for spatial and demographic expansion (Table 4). Most introduced
383 populations showed a unimodal distribution for both expansion models with non-significant
384 SSD and raggedness indices (Table 4), supporting the presence of spatial and demographic
385 expansions. However, the Northern Territory population had significant or marginally
386 significant p-values for SSD and raggedness values for both expansion models indicating
387 poor support (spatial expansion: SSD *p*-value = 0.03, raggedness *p*-value = 0.05;
388 demographic expansion: SSD *p*-value = 0.07, raggedness *p*-value = 0.03).

389 Discussion

390 This study uses mitochondrial genome sequences to study the evolutionary and
391 phylogenetic relationships of the iconic invasive cane toad. Here, we constructed the first
392 reference mitochondrial genome of *R. marina* and placed this species within the order
393 Anura. We utilised RNASeq-derived mitogenomes to illustrate a greater number and
394 diversity of haplotypes in mtDNA present in Australia and Hawai'i, in contrast to the
395 surprisingly low genetic diversity previously described using the *ND3* gene alone. Our
396 conclusions are further supported by evidence of demographic and spatial expansion in

397 invasive cane toad populations. This study provides new insights into the diversity of native
398 cane toads and how they have evolved along their invasion trajectory.

399 ## Phylogenetic inference

400 The phylogenetic relationships of toads within Anura are understudied. More specifically,
401 the cryptic *Rhinella* species complex remains poorly understood (Pereyra et al., 2021). Zhang
402 et al. (2013) attempted to study the Anuran phylogeny using partial mitochondrial genomes.
403 However, no *Rhinella* species were included in their study. Currently, the evolutionary
404 relationship of *Rhinella* species in the order Anura has yet to be fully documented using
405 complete mitochondrial genome or even nuclear genes and/or mitochondrial genes. To
406 address this knowledge gap, we generated the first complete mitochondrial genome from
407 any *Rhinella* species and investigated the phylogenetic relationship of *Rhinella* to other
408 Anurans. Although the topology of our tree confirmed previous hypotheses (e.g., in line with
409 Feng et al. (2017), *Rhinella* formed a sister clade with *Anaxyrus*), it also yielded different
410 conclusions in some aspects. For example, Feng et al. (2017) built a tree for frog species
411 using 95 unlinked nuclear genes and suggested that the genus *Bufo* is monophyletic with
412 respect to *Duttaphrynus*. In contrast, our whole-mitochondrial genome phylogenetic
413 analysis found that the genus *Bufo* is a subclade of *Duttaphrynus*. Our study provides one of
414 the most comprehensive phylogenetic analyses of the relationships within the order Anura
415 to date and provides a starting point for future phylogenetic analyses within the genus
416 *Rhinella*.

417 Choosing to use a complete dataset of a few selected loci versus a large supermatrix with
418 missing data for phylogenetic inference remains a controversial issue (see for review

419 (Lemmon, Brown, Stanger-Hall, & Lemmon, 2009; Roure, Baurain, & Philippe, 2013; Wiens &
420 Morrill, 2011)). Typically, phylogenetic trees are generated using single or multiple nuclear
421 genes, single or multiple nuclear genes plus mitochondrial genes, or multiple nuclear genes
422 with morphological trait matrices, but the use of complete mitochondrial genomes for
423 phylogenetic inference is rare due to resource limitations. Missing data in phylogenetic
424 analyses can bias phylogenetic calculation and tree branch lengths (Lemmon et al., 2009;
425 Xia, 2014). Nonetheless, contrasting findings have also suggested that when a sufficient
426 number of loci and taxa are included, phylogenetic signals remain consistent regardless of
427 whether or not the dataset includes missing data (Molloy & Warnow, 2018; Shavit Grievink,
428 Penny, & Holland, 2013). Despite using complete mitochondrial genomes in this study, 11%
429 of branches remained statistically unsupported, and the genera *Bufo*, *Bufotes*, *Anaxyrus* and
430 *Rhinella* in the Bufonidae family are also not well-supported. This result suggests that the
431 resolution power of the complete mitochondrial genome may not be sufficient, and/or
432 more representative sampling of different anuran species may be needed to provide more
433 robust support for statistical calculations and improve our understanding of the phylogeny.

434 ## Comparison between RNASeq & WGS results

435 The use of transcriptomic data to build mitochondrial genomes has been suggested as a
436 cost-effective alternative to long PCRs or direct sequencing of total DNA (Forni et al., 2019),
437 but it is important to ensure that the inferred polymorphisms accurately reflect genetic
438 variation. In our study, comparing mtDNA data obtained from WGS and RNASeq, we found
439 discrepancies in multiple nucleotide positions, all of which were in rRNA, tRNA and the
440 control region, which could be the result of post-transcriptional modifications or RNA
441 editing which are well-recognised phenomena across multiple taxa (Levanon et al., 2004;

442 Porath, Knisbacher, Eisenberg, & Levanon, 2017; St Laurent et al., 2013). While common
443 substitutions such as adenosine to inosine (A-to-I) deamination and cytidine to uridine (C-to-
444 U) pyrimidine exchange were found, we also observed less common substitutions (e.g.
445 thymine to cytidine (T-to-C) that have been previously documented in another toad species,
446 *Xenopus tropicalis* (Zaranek, Levanon, Zecharia, Clegg, & Church, 2010). These processes are
447 important for creating multiple transcript isoforms and gene regulation (Tang, Fei, & Page,
448 2012) and are known to be associated with adaptation (Duan, Dou, Luo, Zhang, & Lu, 2017;
449 Duan et al., 2021). RNA editing of the tRNA anticodon recognition site would extend the
450 codon matching ability and further increase the variation of the resulting translated
451 proteins. Although we did not observe nucleotide differences in position 34 or position 37 of
452 tRNA, which are typically recognised for deamination of adenosine to inosine which can be
453 paired with A, C or U residues (Jackman & Alfonzo, 2013), we did find changes in the D arm
454 of tRNA (Aspartic acid and Lysine), the T arm of tRNA (Phenylalanine) and the acceptor stem
455 of tRNA (Tryptophan) (Figure S2), which are critical for the recognition of aminoacyl tRNA
456 synthetases to bind appropriate amino acids to its designated tRNA (Ganesh & Maerkl,
457 2022). Failure to form a tRNA-amino acid complex could inhibit the use of a specific amino
458 acid in subsequent protein translation. Further proteome analysis is required to determine if
459 these changes affect the protein translation machinery.

460 Post-transcriptional modifications were more diverse in the native populations than in the
461 invasive populations. In particular, one individual from French Guiana displayed
462 discrepancies in the control region between WGS and RNASeq data, all of which were
463 transitions. This observation might result from tissue-specific expression in spleen or RNA

464 editing. Studies of RNA editing in mtDNA have primarily focused on protein-coding genes
465 and tRNAs, with less research conducted on the control region of the mtDNA, which is
466 responsible for regulating RNA and DNA synthesis. While the implications of potential RNA
467 editing in cane toads remain unclear, these events may contribute to diversity at a
468 transcriptional and translational level. The low diversity of post-transcriptional modification
469 in the invasive populations might be the result of selective sweep for favourable haplotypes
470 with low activity of RNA-editing enzymes.

471 NUMTs, or nuclear insertions of mitochondrial DNA fragments (Lopez, Yuhki, Masuda, Modi,
472 & O'Brien, 1994), can potentially present another source of genetic variation and contribute
473 to false-positive signals of variation in nuclear data (Song, Buhay, Whiting, & Crandall, 2008).
474 NUMTs have been reported in many different taxa including vertebrates, plants and protists
475 (Hazkani-Covo, Zeller, & Martin, 2010). They vary in size, ranging from minimal insertions to
476 hundreds of kilobases and tend to be longer in species with larger nuclear genomes
477 (Hazkani-Covo et al., 2010; Ko & Kim, 2016). Despite NUMTs generally being perceived as
478 non-functional due to their ability to shift reading frames, the presence of in-frame stop
479 codons and the use of different genetic codes, the transcription of NUMTs into non-coding
480 RNA molecules can be captured by RNASeq and potentially falsely interpreted as
481 mitochondrial variation among or within individuals. NUMTs studies on amphibian species
482 are limited. In *Xenopus tropicalis*, over 10kb of NUMTs has been found in the nuclear
483 genome (Hazkani-Covo et al., 2010). Investigation of the presence/absence of NUMTs in
484 cane toad nuclear genome and the potential expression of NUMTs is required to distinguish

485 between genuine mtDNA variation within individuals and false-positive signals caused by
486 incorporating nuclear genetic data into mitochondrial genetic analyses.

487 A search for NUMTs in the published cane toad draft genome (Edwards et al., 2018) yielded
488 only matches with simple repeat regions within the mtDNA control region. In other species,
489 NUMTs originate across the entire mtDNA genome (Dayama, Zhou, Prado-Martinez,
490 Marques-Bonet, & Mills, 2020; Wei et al., 2022). Following the repeat-masking of the draft
491 nuclear genome, we found no evidence of NUMTs suggesting that the putative NUMTs were
492 just low-complexity regions in the nuclear genome. Similar to other gene-searching methods
493 such as BUSCO, the accuracy of the search algorithm heavily depends on the contiguity of
494 the genome and the divergence of the query from the database subjects. Given the high
495 number of contigs in the cane toad draft genome, potential NUMTs hits may have been
496 missed through breakpoints. Therefore, it is important to develop a cane toad genome
497 assembly with higher contiguity to improve our ability to investigate NUMTs. Additionally,
498 ancient NUMTs may be too divergent to be detected by the methods employed, but NUMTs
499 with such low identity would not interfere with mtDNA analysis.

500 It is worth noting that the consensus sequences generated from the bioinformatic pipelines
501 used in this study represented the most common nucleotide at positions where multiple
502 nucleotides were found. Although these discrepancies could result from sequencing errors,
503 our filtering is likely to have prevented this. As discussed above, RNA editing or NUMTs
504 could have resulted in putative polymorphisms within individuals in our RNASeq dataset. It
505 is also possible that in some cases, heteroplasmy could have caused these putative

polymorphisms. While determining the cause of this phenomenon is beyond the scope of this study, it is unlikely that this has affected population-level statistics.

Population genetic analyses

Interestingly, both the haplotype network (Figure 4) and the multimodal mismatch distribution (Figure S1) indicate the presence of multiple genetically divergent lineages within native populations. Notably, individuals within each lineage are intermixed, rather than being grouped by geographical locations. For instance, despite an 80 km geographical separation, haplotypes H4 and H7 are found in the same lineage while haplotypes H12 and H11 were collected only 9 m apart but belong to different lineages. Native-range cane toads can travel 60-180 m per move for sheltering and foraging purposes (Shine et al., 2021). However, the overall dispersal rates are quite low, as the toads ultimately tend to return to the same shelters (DeVore, Shine, & Ducatez, 2021). Consequently, the possibility of hybridisation between toads from these distinct lineages exists. However, a recent study in South and Central America which utilised ddRAD sequencing has offered a different perspective on the phylogenetic relationship of cane toads. This study revealed toads from the coastal area formed two sister clades, distinct from the clade formed by toads from the rainforest regions (Mittan-Moreau et al., 2022). It becomes crucial to ascertain the precise populations of cane toads from the native range that were used as founding individuals, especially given the limited comprehensive description in prior research (Easteal, 1981). In Shine et al. (2021), cane toads from the range-edge regions (Northern Territory and Western Australia) exhibited similar dispersal patterns to native toads in one of the coastal sites. This suggests the adaptation of cane toads in hot and arid environments is likely facilitated by the standing genetic variation(s) inherited from the progenitors utilising dry,

529 saline coastal beach environments (DeVore et al., 2021), rather than relying solely on the
530 genetic adaptation and selection of novel mutation(s). A more thorough investigation into
531 these distinct lineages becomes imperative for unravelling the mechanisms underlying the
532 adaptations of cane toads in Australia.

533 Our study challenges the assumption of low genetic diversity in Hawai'ian and Australian
534 populations. Slade and Moritz (1998) reported a unique haplotype at *ND3* within Australian
535 populations, in comparison with multiple haplotypes in native populations in Central and
536 South America. This result supported the presence of founder effects following the
537 introduction of cane toads into Hawai'i and Australia. Here, we revealed two additional,
538 previously undocumented, *ND3* haplotypes (Hap2 and Hap3) in Hawai'ian and Australian
539 populations, each of which differed by only one nucleotide from the previously identified
540 haplotype (Hap1) (Figure 5). In comparison to our analysis using nearly complete
541 mitogenomes discussed above, this analysis highlights that a single gene is not sufficient to
542 accurately infer genetic diversity.

543 The significant decrease in haplotype diversity and nucleotide diversity observed in both
544 Hawai'ian and Australian populations, compared to native populations, does support that
545 genetic bottlenecks and founder effects occurred post-introduction (Table 1, Figure 6). This
546 finding is consistent with previous studies on this species using nuclear genetic markers
547 including SNPs, microsatellite markers, and MHC class I and II loci (Lillie et al., 2016; Lillie et
548 al., 2014; Selechnik, Richardson, Shine, DeVore, et al., 2019). In this study, all three
549 neutrality indices showed strong signals of population expansion and the observed data
550 fitted to both demographic expansion and spatial expansion models in most invasive

551 sampling sites (Table 4). This conclusion is supported by the star-like shaped topology of the
552 haplotype network created using the nearly-complete mitochondrial genome dataset.
553 Despite reduced diversity in the introduced range, the degree of the decrease is less than
554 previously reported (Leblois et al., 2000). Additionally, the star-shaped topology of the
555 network containing the introduced haplotypes, the absence of native samples here, and the
556 significant negative Fu's *Fs* values indicate that many of the singleton haplotypes discovered
557 in Hawai'i and Australia may be the products of post-introduction mutations. Alternatively,
558 due to the limited sample sizes in the native range, it is possible that any introduced
559 haplotypes exist but were not included in our sample set for analysis.

560 We observed a curvilinear pattern of genetic diversity indices across Australian populations
561 (from Queensland to Western Australia), a pattern also found in spleen gene expression
562 (Selechnik, Richardson, Shine, Brown, et al., 2019), limb length (Hudson, McCurry, Lundgren,
563 McHenry, & Shine, 2016), spleen and body mass (Gregory P. Brown, Kelehear, Shilton,
564 Phillips, & Shine, 2015). The mechanism behind this reduction of genetic diversity in
565 Northern Territory toads remains unclear, but it may be due to demographic processes that
566 reduce the effective population size and increase genetic drift (Nei & Tajima, 1981), genetic
567 hitchhiking that removes neutral polymorphic sites under positive selection (Fay & Wu,
568 2000) or directional selection due to harsh climate (Selechnik, Richardson, Shine, DeVore, et
569 al., 2019), possibly in combination with dispersion-driven spatial sorting at the invasion front
570 (Clarke, Shine, & Phillips, 2019). Despite our finding that the Northern Territory and Western
571 Australia populations were not genetically differentiated, it is likely that they have
572 experienced different local evolutionary forces and demographic processes. Northern

573 Territory toads, unlike other populations, only exhibited demographic expansion and not
574 spatial expansion, as supported by lower levels in all statistical measures.

575 Our results demonstrate significant differentiation between native and introduced
576 populations (Table 3). We identified three genetic clusters: 1) native toads, 2) Hawai'i and
577 Queensland toads (*Fst*: 0.016, $p = 0.056$) and 3) Northern Territory and Western Australia
578 toads (*Fst*: -0.012, $p = 0.72$), consistent with previous results using SNPs from the nuclear
579 genome (Selechnik, Richardson, Shine, DeVore, et al., 2019). It is interesting that the
580 Queensland toads are not differentiated from those sampled in Hawai'i but differ from
581 toads sampled in the Northern Territory and Western Australia. Queensland toads have
582 been separated from their Hawai'ian conspecifics for almost 90 years but do share similar
583 environmental conditions (Selechnik, Richardson, Shine, DeVore, et al., 2019). Northern
584 Territory and Western Australian toads suffer from reduced genetic diversity compared to
585 Queensland toads, so they have likely experienced more genetic drift, which may explain
586 this difference. However, it is also possible that the striking reduction in precipitation and
587 increase in temperature experienced by Northern Territory and Western Australian toads
588 compared to those from Queensland has resulted in selective differences in the
589 mitogenome between these populations. In support of this possibility, significant negative
590 Fu's *Fs* can indicate the presence of selection.

591 ## Conclusion

592 Our study used the whole mtDNA genome as a genetic marker to investigate the evolution
593 of cane toads across their invasion trajectory from the native range to Australia, adding to
594 our knowledge of changes to genetic diversity as cane toads have been serially introduced

595 to multiple locations. Unsurprisingly, we found reduced genetic diversity in introduced
596 populations, suggesting that genetic bottlenecks have occurred in these populations.
597 Despite this reduction, our data indicate greater genetic diversity in introduced populations
598 than has been previously described using mtDNA. We identified signatures of demographic
599 and spatial expansion in most introduced localities we sampled, consistent with ongoing
600 invasion. We described 46 haplotypes in introduced populations, mostly consisting of single
601 base-pair changes, suggesting evolution following introduction. By the comparison of WGS-
602 and RNASeq-derived mitogenomes, we have identified the presence of post-transcriptional
603 modification or RNA editing, which may supply additional genetic diversity upon which
604 selection can act.

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Author Contributions

KC, RS, RJE, and LAR developed this project. JLD and SD conducted field work. LAR conducted DNA extractions. KC, TGA, RJE, and LAR analysed the data. All authors contributed to data interpretation and writing.

Data availability

The haplotype sequences will be available on the NCBI public database once the manuscript is accepted.

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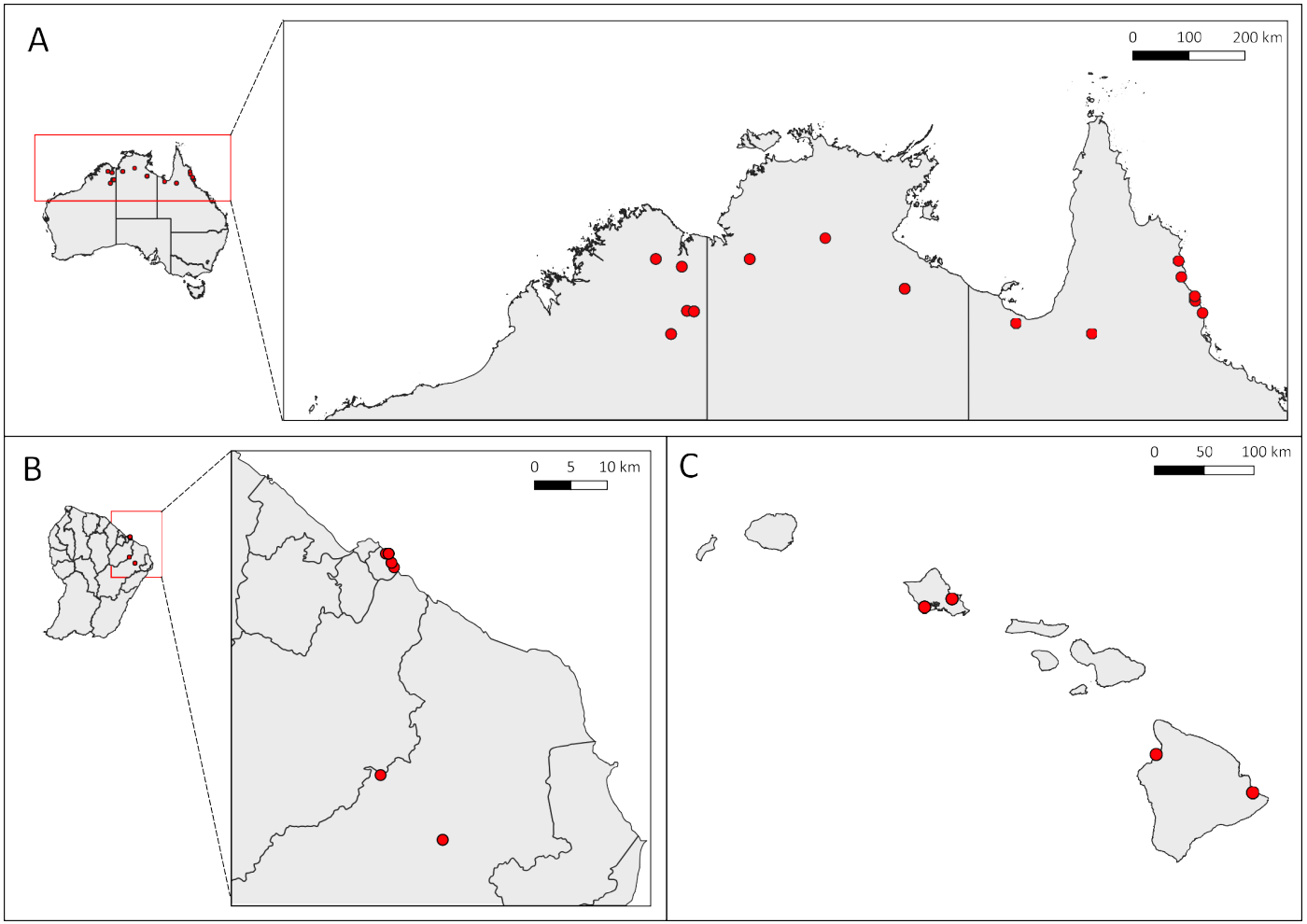
Tables and Figures

Figure 1: Sampling locations of the cane toad (*Rhinella marina*) in (A) Australia and (B) French Guiana (the native range) and (C) Hawai'i. Red dots in French Guiana, Hawai'i and Australia indicate sample collection sites.



935 Figure 2: Mitochondrial genome structure of *R. marina*. Genes, GC skew and GC content
936 were shown in the inner circle, middle circle and outer circle respectively. The innermost
937 circle indicates the negative strand and the outer circle indicates the positive strand. Genes
938 are coloured by type: red, tRNA; pink, rRNA; blue, coding sequence. Starting and stop
939 positions of each gene are described in Table S3.

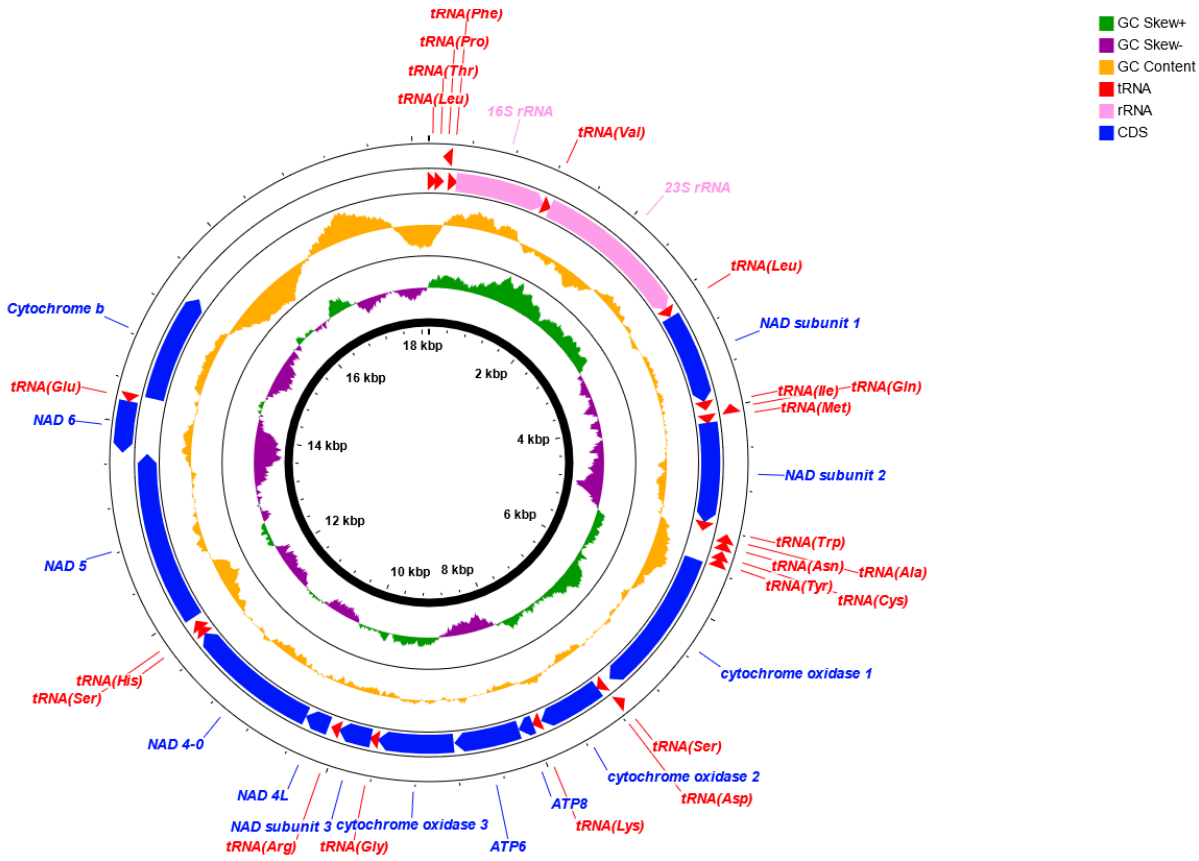


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Figure 3: Maximum-likelihood phylogenetic tree of the complete mitochondrial genomes of 203 Anuran species downloaded from the NCBI BioProject public database. Five non-Anuran species from Caudata and Caeciliidae were used as outgroup (Table S3). The dendrogram was constructed using IQ-Tree multicore version 2.2.2.6. Branch lengths represent genetic distances. Ultrafast bootstrap and SH-aLRT branch tests were performed with 10,000 replications. Branches with SH-aLRT values ≤ 80% or UFboot values ≤ 95% were labelled with red dots. The leftmost and rightmost panels display zoomed upper and lower parts of the dendrogram, respectively. The cane toad (*Rhinella marina*) labelled with a red arrow.

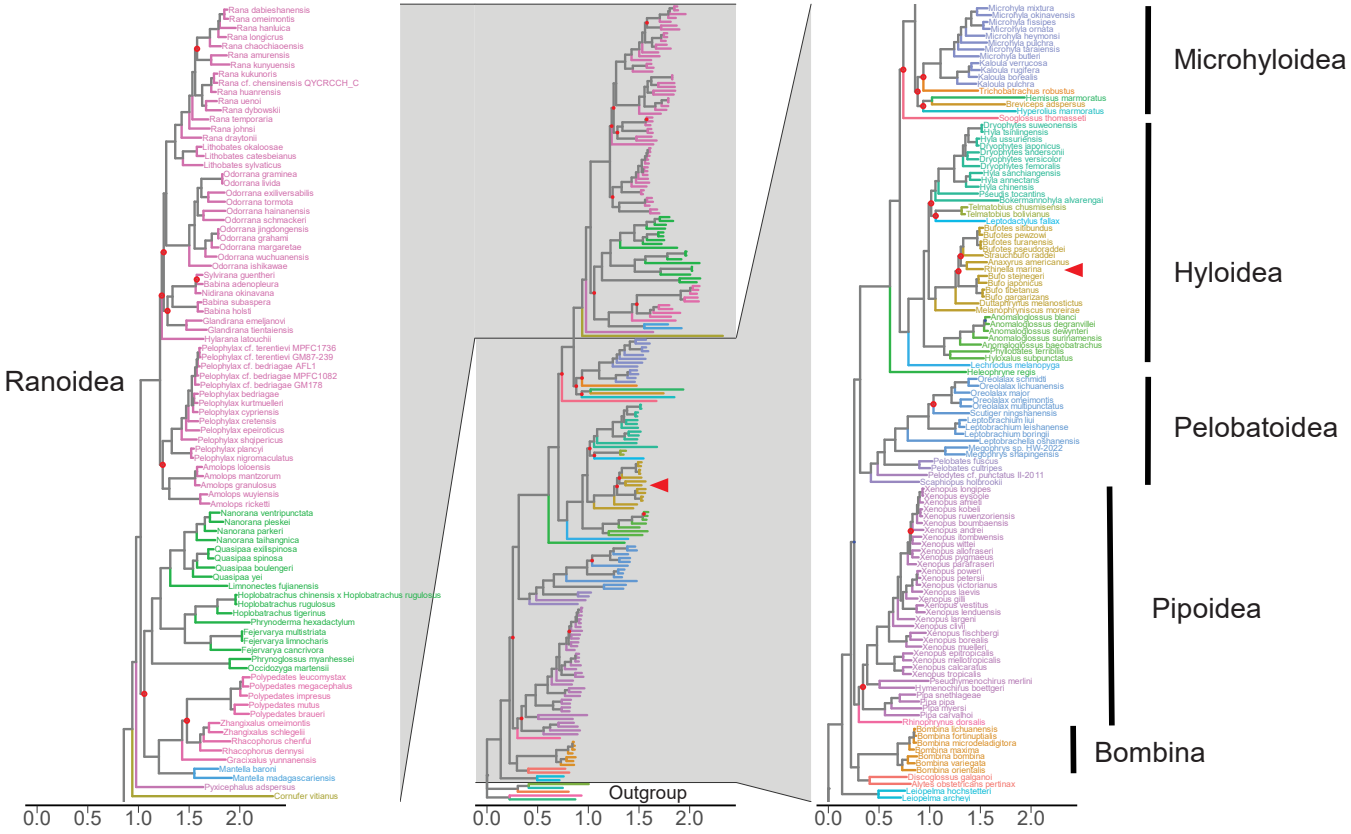


Figure 4: The haplotype network illustrating the relationships among 132 individuals from French Guinea, Hawai'i, and Australia based on near-complete mitochondrial genomes. Each sample location is represented by a different colour. A black solid circle represents a putative haplotype that was not directly sampled. The red number adjacent to a line indicates the number of nucleotide substitutions between haplotypes, while the absence of a number indicates a single nucleotide substitution.

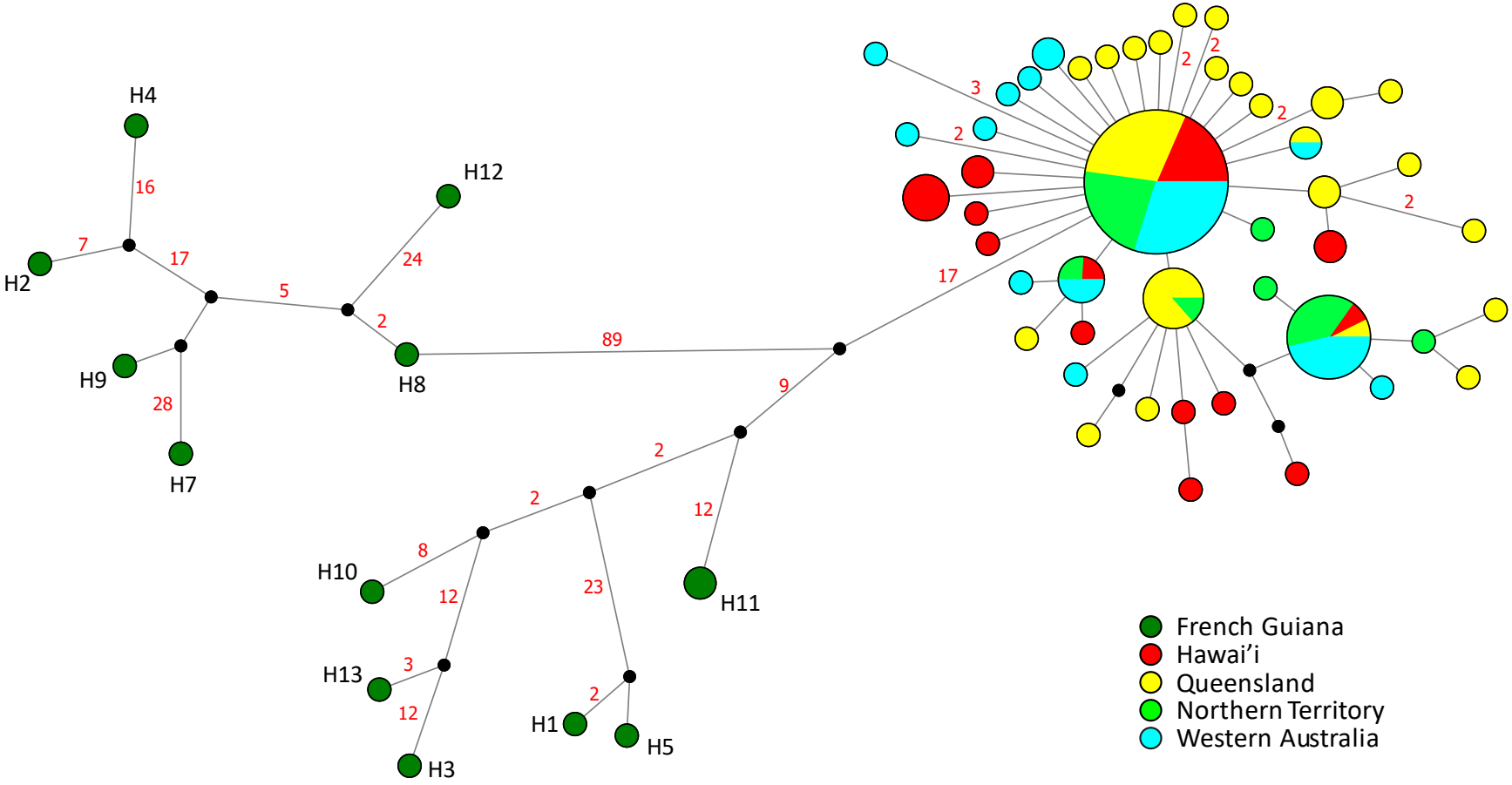


Figure 5: Haplotype network of a 510 bp segment of ND3 with flanking tRNAs, a total of 223 samples, including samples from this study, Slade and Moritz (1998) and Acevedo et al. (2016). Sample locations are represented by different colours. A black solid circle represents a putative haplotype that was not directly sampled. The red number adjacent to a line indicates the number of nucleotide substitutions between haplotypes, while the absence of a number indicates a single nucleotide substitution. Samples are separated by geographical locations on each side of the Andes Mountains in South America (Acevedo et al., 2016). Toads on the west side of the Andes are now known as *Rhinella horribilis*.

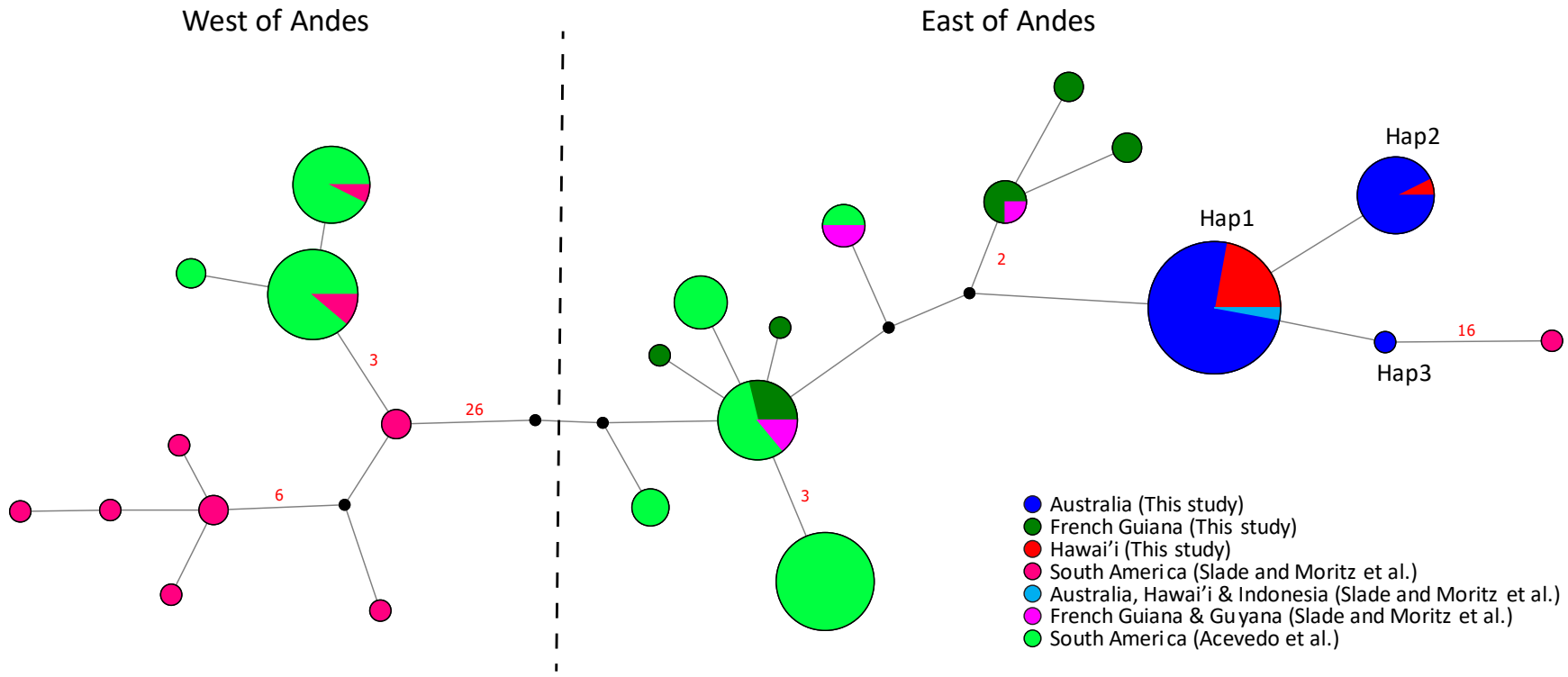
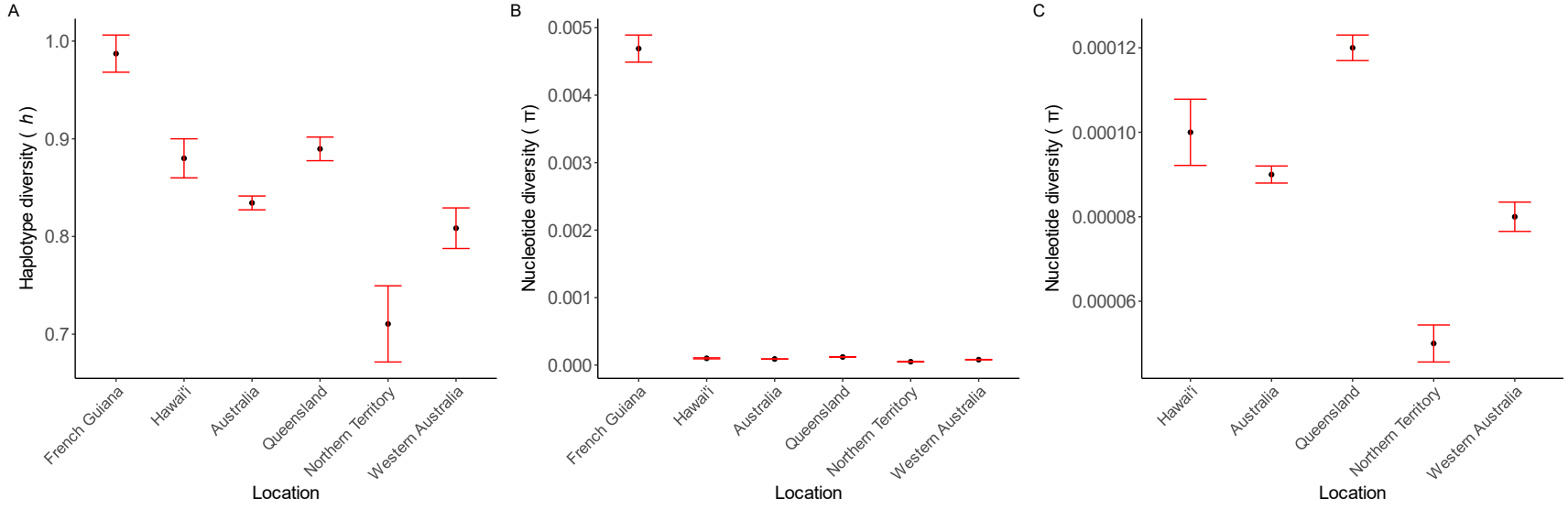


Figure 6: Haplotype diversity (A) and nucleotide diversities (B and C) of native population and introduced populations.



962 Table 1: Population genetics statistics of native range population (French Guiana) and introduced populations (Hawai'i and Australia).

	N	**Polymorphic sites**	***H***	**π ± SD**	***h* ± SD**	**HR**
French Guiana	13	205	12	0.00469 ± 0.00037	0.987 ± 0.035	12
Hawai'i	25	11	13	0.00010 ± 0.00002	0.880 ± 0.051	10.7
Australia	94	40	36	0.00009 ± 0.00001	0.834 ± 0.035	12.5
Queensland	42	27	23	0.00012 ± 0.00001	0.890 ± 0.040	13.5
Northern Territory	20	5	7	0.00005 ± 0.00001	0.711 ± 0.089	6.4
Western Australia	32	16	13	0.00008 ± 0.00001	0.809 ± 0.06	9.6

N: Number of samples; *H*: Number of haplotypes; π: nucleotide diversity; *h*: haplotype diversity; HR; haplotype richness.

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964 Table 2: Analysis of molecular variance (AMOVA) of all populations.

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Source of variation	*df*	Sum of squares	% of variation	Fixation indices
All populations				
Among groups	2	482.96	64.49	
Among populations within groups	2	2.91	-0.82	
Within populations	127	579.35	36.33	F_{ST} = 0.64***

***: $p < 0.001$

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Table 3: Population differentiation (pairwise *Fst*) between native range and introduced ranges (Hawai'i and Australia) (below the diagonal line) The associated *p*-values were shown above the diagonal line.

	French Guiana	**Hawai'i**	**Queensland**	**Northern Territory**	**Western Australia**	**Australia**
French Guiana		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Hawai'i	0.58		0.06	0.01	0.01	0.02
Queensland	0.67	0.02		0.009	0.003	0.34
Northern Territory	0.55	0.05	0.04		0.72	0.54
Western Australia	0.63	0.03	0.03	-0.01		0.8
Australia	0.79	0.02	0.002	-0.004	-0.006	

970 Table 4: Demographic estimators of native range population (French Guiana) and introduced populations (Hawai'i and Australia).

			Demographic expansion		Spatial expansion	
	Fu's *Fs*	**R_2**	**SSD**	**Raggedness**	**SSD**	**Raggedness**
French Guiana	1.29 (p = 0.66)	0.18 (p = 0.891)	0.03 (p = 0.19)	0.03 (p = 0.39)	0.03 (p = 0.08)	0.03 (p = 0.55)
Hawai'i	-8.78 (p < 0.001)	0.08 (p = 0.02)	0.01 (p = 0.44)	0.080 (p = 0.24)	0.01 (p = 0.31)	0.08 (p = 0.21)
Australia	-27.92 (p < 0.001)	0.02 (p < 0.001)	0.002 (p = 0.30)	0.067 (p = 0.16)	0.002 (p = 0.20)	0.07 (p = 0.14)
Queensland	-23.04 (p < 0.001)	0.03 (p < 0.001)	0.001 (p = 0.70)	0.05 (p = 0.38)	0.002 (p = 0.66)	0.05 (p = 0.42)
Northern Territory	-3.91 (p < 0.001)	0.10 (p = 0.072)	0.03 (p = 0.07)	0.21 (p = 0.03)	0.03 (p = 0.03)	0.21 (p = 0.05)
Western Australia	-8.950 (p < 0.001)	0.05 (p = 0.001)	0.003 (p = 0.58)	0.08 (p = 0.34)	0.003 (p= 0.35)	0.08 (p = 0.26)

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