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Population genomics and sexual signals support reproductive character displacement in *Uperoleia* (Anura: Myobatrachidae) in a contact zone

Frederick R. Jaya[1] | Jessie C. Tanner[2] | Michael R. Whitehead[3] | Paul Doughty[4] | J. Scott Keogh[5] | Craig C. Moritz[5] | Renee A. Catullo[2,5]

[1]Australian Institute for Microbiology and Infection, University of Technology Sydney, Ultimo, New South Wales, Australia

[2]Centre for Evolutionary Biology, School of Biological Sciences, University of Western Australia, Crawley, Western Australia, Australia

[3]School of BioSciences, University of Melbourne, Parkville, Victoria, Australia

[4]Western Australian Museum, Perth, Western Australia, Australia

[5]Centre for Biodiversity Analysis, Ecology & Evolution, Research School of Biology, Australian National University, Acton, Australian Capital Territory, Australia

Correspondence
Renee A. Catullo, Centre for Evolutionary Biology, School of Biological Sciences, University of Western Australia, Crawley, WA, Australia.
Email: renee.catullo@uwa.edu.au

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Abstract

When closely related species come into contact via range expansion, both may experience reduced fitness as a result of the interaction. Selection is expected to favour traits that minimize costly interspecies reproductive interactions (such as mismating) via a phenomenon called reproductive character displacement (RCD). Research on RCD frequently assumes secondary contact between species, but the geographical history of species interactions is often unknown. Population genomic data permit tests of geographical hypotheses about species origins and secondary contact through range expansion. We used population genomic data from single nucleotide polymorphisms (SNPs), mitochondrial sequence data, advertisement call data and morphological data to investigate a species complex of toadlets (*Uperoleia borealis*, *U. crassa*, *U. inundata*) from northern Australia. Although the three species of frogs were morphologically indistinguishable in our analysis, we determined that *U. crassa* and *U. inundata* form a single species (synonymized here) based on an absence of genomic divergence. SNP data identified the phylogeographical origin of *U. crassa* as the Top End, with subsequent westward invasion into the range of *U. borealis* in the Kimberley. We identified six F_1 hybrids, all of which had the *U. borealis* mitochondrial haplotype, suggesting unidirectional hybridization. Consistent with the RCD hypothesis, *U. borealis* and *U. crassa* sexual signals differ more in sympatry than in allopatry. Hybrid males have intermediate calls, which probably reduces attractiveness to females. Integrating population genomic data, mitochondrial sequencing, morphology and behavioural approaches provides an unusually detailed collection of evidence for reproductive character displacement following range expansion and secondary contact.

KEYWORDS
mitochondrial genome, range expansion, reproductive interference, speciation, unidirectional hybridization

Frederick R. Jaya and Jessie C. Tanner shared first author.

1 | INTRODUCTION

When two closely related species with similar ecological niches come into contact through range expansion, both may experience reduced fitness as a result of the interaction (Brown & Wilson, 1956; Pfennig & Pfennig, 2009). Reduced fitness may be caused by reduced access to resources or lower rates of successful reproduction. The latter includes "mismating" with heterospecifics in systems where hybridization reduces offspring viability or fertility (Cooley, 2007; Liou & Price, 1994). As a result, one or both species may experience a shift in traits in response to selection acting to minimize costly interspecies interactions, in a process termed reproductive character displacement (RCD; Cooley, 2007; Pfennig & Pfennig, 2009). The process of RCD is often inferred using the pattern of variation in reproductive traits it generates (Gerhardt, 1994; Lemmon, 2009; Pfennig & Pfennig, 2009). Specifically, reproductive characters of two species should be more divergent in geographical areas where both species occur (sympatry) than in areas where either one occurs alone (allopatry).

The biogeographical context of RCD is important to understanding the directionality and strength of reproductive interference. In many cases of RCD, a single species is fully sympatric with another, while this second, more widespread species has portions of its range that are allopatric and sympatric (Cooley et al., 2006; Geyer & Palumbi, 2003; Higgie et al., 2000; Kulathinal & Singh, 2000; Waage, 1979). It is this second species in which character displacement may be detected by comparing mating traits in the two areas of the range. The most common hypothesis in these circumstances is that these species arose via vicariant speciation, with secondary contact driving RCD. This scenario explicitly requires that one species is an invader into the sympatric area. The invading species will have a smaller number of individuals at the invasion front as they enter the distribution of the other species (e.g., Grayson & Johnson, 2018). Therefore, the relative costs of hybridization would be unequal (allee effects; Bordenstein et al., 2000; Contarini et al., 2009; Stephens et al., 1999; Tynkkynen et al., 2004); if the already-present species is abundant at the time of invasion, most females will easily find conspecific mates, while females of the invading species must identify the few conspecific males among the much larger number of heterospecifics (Kishi, 2015; Kyogoku & Nishida, 2012). The invading species is expected to suffer strong reproductive interference because low population sizes at the invasion front lead to a higher probability of mismating, increasing the likelihood of local extinction (Kyogoku & Wheatcroft, 2020), and it is in this invading species that RCD is likely to occur. Therefore, the eco-evolutionary and geographical context of diversification and invasion are important to understanding how speciation and secondary contact result in RCD, and which species should show evolution of mating traits.

Frogs have been productive models for studying RCD because females primarily use the acoustic signals of males to identify, discriminate among and locate appropriate mates (Gerhardt & Huber, 2002; Wells, 2010). Females typically have preferences for the temporal and spectral properties of male signals and exert sexual selection through mate choice (Bodnar, 1996; Gerhardt & Brooks, 2009; Robertson, 1990; Tanner et al., 2017; Tanner & Bee, 2019). This reliance on acoustic signals for species identification and assessment of mate quality puts pressure on closely related, co-occurring species to evolve sufficiently distinct acoustic signals to avoid misidentification and heterospecific matings (Gerhardt, 1994; Höbel & Gerhardt, 2003; Lemmon, 2009). This is especially true where hybrids have reduced fitness (Arnold & Hodges, 1995; Arnold & Martin, 2010; Gerhardt et al., 1994; Lemmon & Lemmon, 2010; Parris, 1999; Pfennig, 2007; Pfennig et al., 2016).

The genus *Uperoleia* (Anura: Myobatrachidae) presents a good opportunity to study species boundaries, range expansion and associated effects on sexual signalling. *Uperoleia*, or "toadlets," are small, sexually size-dimorphic (males <3 cm, females <4 cm) burrowing frogs distributed across northern and eastern Australia. The genus comprises 29 species that extensively overlap in distribution, with molecular evidence supporting widespread hybridization among species in the past (Catullo, Doughty, et al., 2014; Clulow et al., 2016). Although closely related species in this clade of small brown frogs are often morphologically similar, they can be delimited using genetic data and differences in their calls (Catullo, Doughty, et al., 2014; Catullo, Lanfear et al., 2014; Doughty & Roberts, 2008).

Uperoleia borealis, *U. crassa* and *U. inundata* form a species complex widely distributed across the Kimberley region of northern Western Australia and the Top End region of the Northern Territory. Current taxonomy suggests *U. borealis* and *U. crassa* are restricted to the Kimberley, whereas *U. inundata* occurs in both the Top End and Kimberley (Figure S1; Catullo & Keogh, 2014). All three species were originally described as distinct taxa in the same publication, based primarily on differences between their advertisement signals, extent of webbing in the toes and subtle differences in patterning (Tyler et al., 1980). However, molecular data recently suggested that *U. crassa* and *U. inundata* may be a single species (Catullo & Keogh, 2014). We hypothesize that the differences between their calls may be attributable instead to RCD in the Kimberley where *U. crassa*/*U. inundata* co-occurs with *U. borealis*. This new hypothesis challenges our understanding of *Uperoleia* taxonomy in the Kimberley region of Australia, where all three putative species are currently believed to live and breed in close proximity. Although there are probably subtle ecological differences between *U. borealis* and *U. crassa*/*inundata*, there is sufficient overlap in choruses to result in reproductive interference (Doughty et al., 2012; Tyler & Doughty, 2013; our pers. obs.).

In the present study, we use a combination of molecular, morphological and behavioural approaches to evaluate whether *U. crassa* and *U. inundata* are a single species, rather than two. Hereafter, we will refer to both as *U. crassa* and separate them into two geographical groups: the Kimberley *U. crassa* sympatric with *U. borealis* and the Top End *U. crassa* allopatric with *U. borealis*. We use genome-wide single nucleotide polymorphisms (SNPs) and mitochondrial DNA markers to investigate the species boundaries and identify interspecific hybrids, elucidate population structure and patterns of genetic diversity, and infer the phylogeographical origin of *U. crassa*. Then,

using recordings of male calls, we test the hypothesis that sympatric populations of *U. crassa* in the Kimberley are undergoing reproductive character displacement due to the presence of the closely related *U. borealis*. Specifically, because pulse traits are important species-recognition signals in many anurans with pulsed signals, particularly in co-occurring, closely related pairs of frogs (Loftus-Hills & Littlejohn, 1971; Pettitt et al., 2013; Schul & Bush, 2002; Schwartz, 1987; Straughan, 1975), we expect to see RCD in calls as a divergence in pulse characteristics.

2 | METHODS

2.1 | Sample selection

Tissue samples from the Western Australian Museum (WAM), South Australian Museum (ABTC), Queensland Museum (QM) and Northern Territory Museum (NTM) were selected to represent the range of known current distributions of the species complex, with a focus on the Kimberley region of sympatry (Appendix S1). Additional samples from the other closely related *Uperoleia* were sequenced to provide phylogenetic context, based on relationships in Catullo and Keogh (2014). Species selected to represent outgroups are close relatives (*Uperoleia micra*, *U. russelli*, *U. saxatilis* and *U. talpa*), none of which are sympatric with *U. borealis* or *U. crassa*. Vouchers and call recordings were from samples registered in collections, with additional call recordings for unvouchered samples by PD and RAC (Appendix S1). Collections were authorized by the Australian National University Animal Ethics Committee and the Western Australian Department of Biodiversity, Conservation and Attraction's Animal Ethics Committee (DEC AEC#2003/02 and subsequent). Collections were made under Western Australian permit nos. FO25000006-13 and SF007216, and Northern Territory permit nos. 36083 and 63130.

2.2 | Genomic data generation

Tissue samples were sent to Diversity Arrays Technology for commercial DNA extraction and genomic sequencing (see Georges et al., 2018 for detailed description of sequencing approach). DNA concentrations were adjusted to 50 ng μl^{-1} per sample and genotyped utilizing DArTseq 1.0 complexity reduction technology (Georges et al., 2018; Sansaloni et al., 2011). This approach uses two restriction enzymes followed by fragment size selection to generate reduced representation genomic libraries. DNA samples were processed in digestion/ligation reactions following Kilian et al. (2012) using the frequent cutter *Pst*I (5′-CTGCA^G-3′), and the rare cutter *Sph*I (5′-GCATG^C-3′), and ligating two different adapters corresponding to the *Pst*I and *Sph*I overhangs. The *Pst*I-compatible adapter was designed to include an Illumina flow cell attachment sequence, sequencing primer sequence and "staggered," varying length barcode region. The reverse adapter contained a flow cell attachment region and an *Sph*I overhang sequence. Ligated fragments containing both *Pst*I and *Sph*I adapters were polymerase chain reaction (PCR)-amplified with an initial denaturation at 94°C for 1 min, followed by 30 cycles of 94°C for 20 s, 58°C for 30 s and 72°C for 45 s and a final extension step at 72°C for 7 min. Equimolar amounts of amplified samples were multiplexed and applied to cBot (Illumina) bridge PCR followed by single-read sequencing on an Illumina HiSeq2000 for 77 cycles.

Sequences generated from each lane were processed using proprietary DArT analytical pipelines. In the primary pipeline, fastq files were processed to filter out poor quality sequences. More stringent selection criteria were applied to the barcode regions to ensure the reliable assignment of sequences to specific samples during the "barcode splitting" step. Approximately 2.5 million sequences per barcode/sample were identified and used in marker calling. Finally, identical sequences were collapsed into "fastqcoll files" and used in the secondary DArT P/L's proprietary codominant SNP marker calling algorithms (DArTsoft14). To avoid the Wahlund effect in our downstream analyses (De Meeûs, 2018), SNPs were identified four times: (i) across outgroup and ingroup taxa for the phylogenetic analysis, (ii) across all *U. borealis* and *U. crassa* for identification of interspecies hybrids, and (iii) across *U. borealis* and (iv) *U. crassa* separately for within-species population genetic analyses.

2.3 | Genomic analyses

Analyses followed a series of steps to assess species status, population structure within species and status of hybrids (details below; Figure S2). First, an SNP-based phylogenetic tree was generated, to provide evolutionary context for diversification in the group. Next, the full set of individuals from both target species were analysed in STRUCTURE (Pritchard et al., 2000) to identify the major population genetic clusters within our data and likely hybrid individuals. Hybrid class (e.g., F_1, backcross) was assessed using NEWHYBRIDS (Anderson & Thompson, 2002). Each population cluster matched one of two major phylogenetic clades (Figures 1 and 2). Each clade was then analysed separately (minus the few individuals identified as hybrids) to assess within-clade population structure and genetic diversity.

2.4 | Filtering protocols

For all population genetic and phylogenetic analyses, SNPs were filtered using the package DARTR 1.8.3 (Gruber et al., 2018) in R 3.5.1 (R Core Team, 2021). Filtering for each data set is outlined in Figure S2. Filtering for each analysis differed based on the theoretical and computational requirements of downstream analyses (e.g., multiple SNPs per fragment retained for estimating heterozygosity, singletons retained for calculating Tajima's *D* or filtering on a higher loci completeness for the species tree analysis). For each data set, the

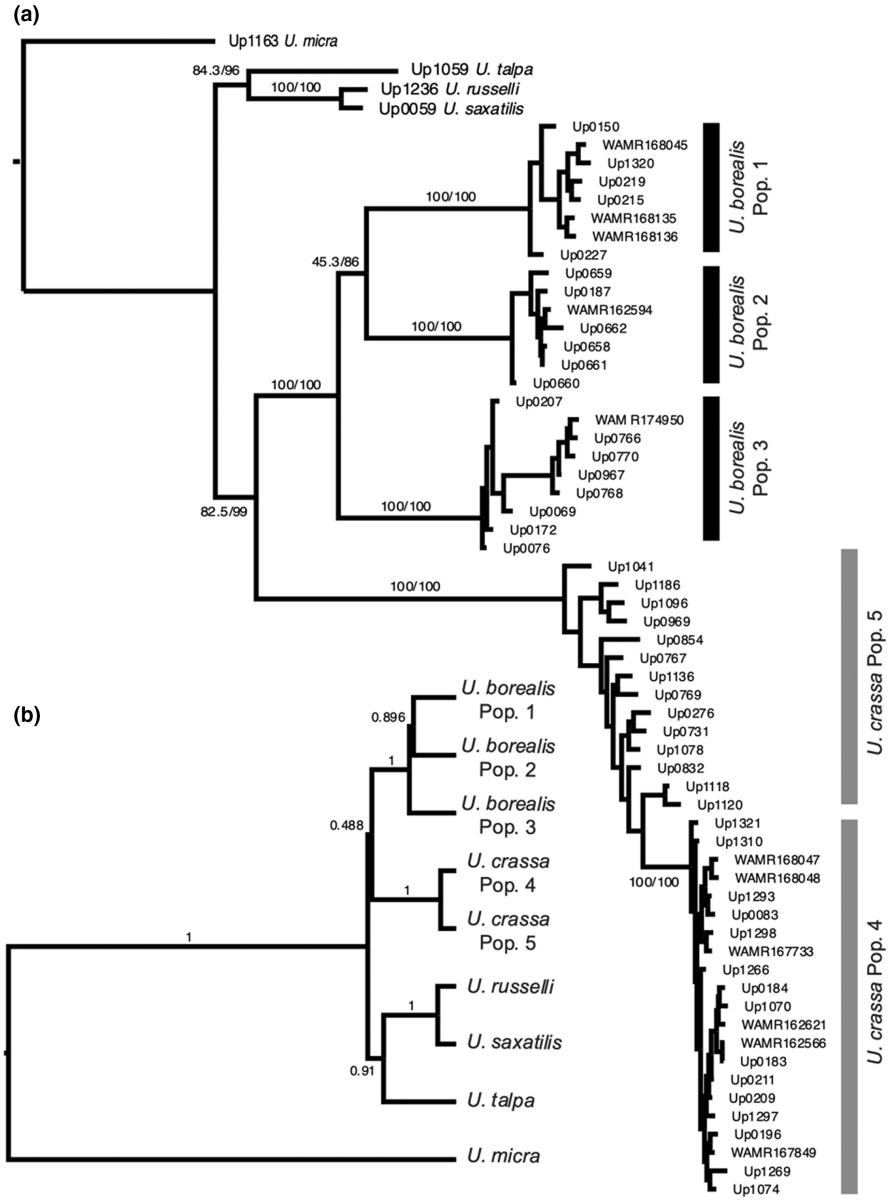

FIGURE 1 (a) Maximum-likelihood phylogeny of SNP data for all nonhybrid individuals of *Uperoleia borealis* and *Uperoleia crassa*, plus representatives of all other closely related species. Values on branches indicate support (SH-like approximate likelihood ratio test [SH-aLRT]/ultrafast bootstrap [UFboot]). (b) Result of the coalescent Bayesian SNAPP analysis, showing high support for species monophyly, but is unable to resolve the interspecific relationships in the clade. Values on branches represent the posterior probability

gl.pcoa function in DARTR 1.8.3 (Gruber et al., 2018) was used to calculate the principal coordinates analysis (PCoA) using filtered SNP data.

2.5 | Phylogenetic analysis of SNP data

Phylogenetic analyses on all nonhybrid individuals in the SNP data set (as identified below) were conducted using maximum likelihood (ML) to provide phylogenetic context between *U. borealis*, *U. crassa* and other closely related *Uperoleia* (following Catullo & Keogh, 2014). The ML phylogeny was constructed for all individuals using IQ-TREE 1.6.12 (Nguyen et al., 2015) with 10,000 ultrafast bootstrap replicates (Minh et al., 2013) and 10,000 replicates of the SH-like approximate likelihood ratio test (SH-aLRT; Guindon et al., 2010). Model selection was completed using MODEL FINDER PRO with ascertainment bias correction (Kalyaanamoorthy et al., 2017).

Species trees were constructed using the SNAPP package (Bryant et al., 2012) in BEAST 2 version 2.5 (Bouckaert et al., 2014). Four individuals per population, as identified in the separate species-level STRUCTURE analyses, were included as "species" and SNPs were independently filtered as detailed in Figure S2. We sampled the mutation rate in the program, set the alpha to 1, beta to 250, lambda to 0.01, sampled a coalescence rate initially set to 10, and used default settings for all other parameters. The SNAPP analysis was run for 50 million generations each, with 10% of the run discarded as burn-in. Convergence was assessed using TRACER and trees were summarized using the TREEANNOTATOR program distributed with BEAST 2.

2.6 | Phylogenetic analysis of mitochondrial data

Genomic sequences for the mitochondrial (mtDNA) genes large ribosomal subunit (16S) and NADH dehydrogenase subunit 2 (*ND2*)

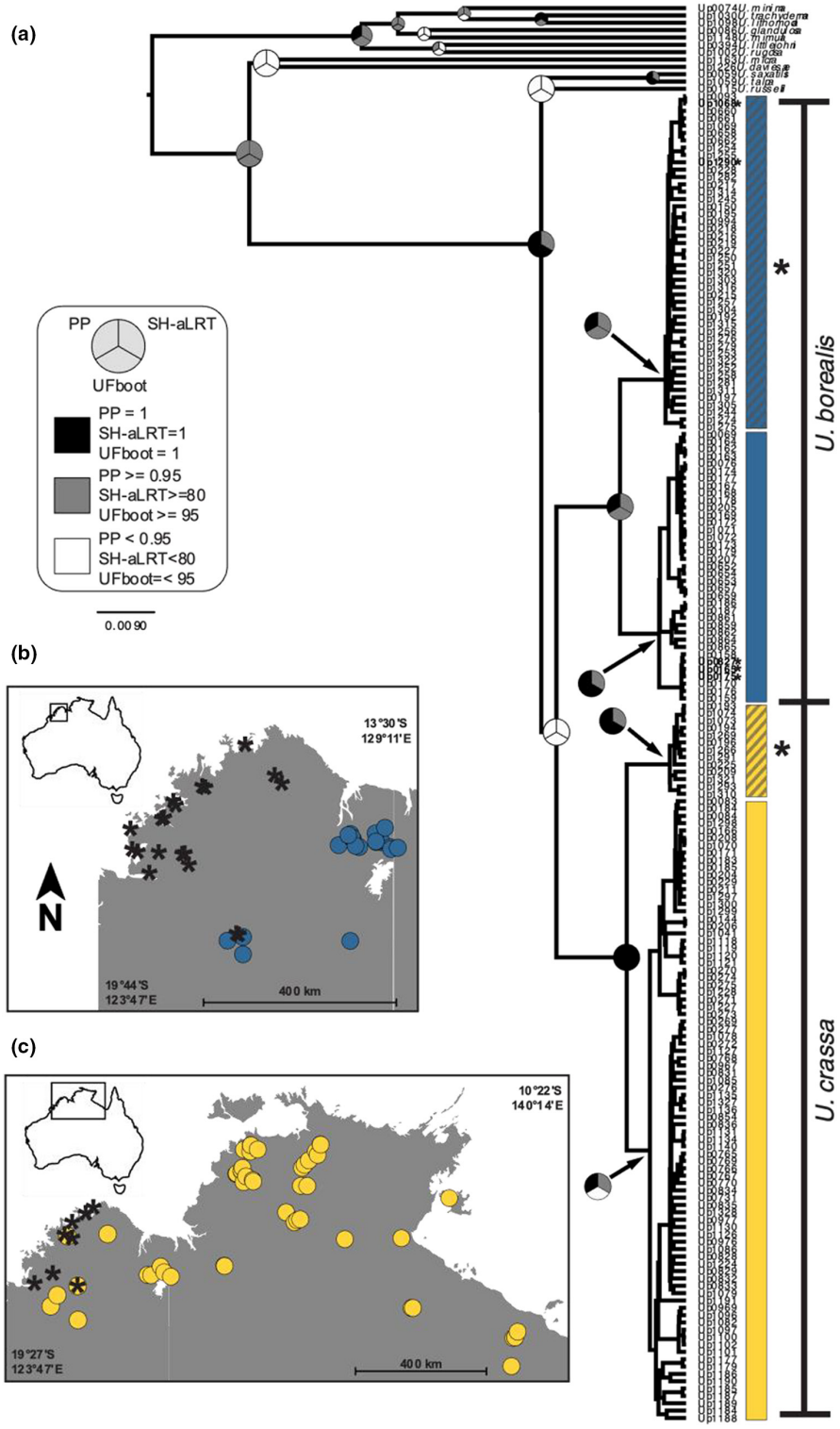

FIGURE 2 Bayesian coalescent phylogeny of the 16S and *ND2* mitochondrial phylogeny (a). Values on nodes represent the support for each node from both the BEAST coalescent analysis and the maximum-likelihood analysis (posterior probability [PP], SH-like approximate likelihood ratio test [SH-aLRT] and ultrafast bootstrap [UFboot]). Individuals identified as F_1 hybrids in the SNP analysis are marked with an asterisk. Distributions of mitochondrial clades are shown for *Uperoleia borealis* (b) and *Uperoleia crassa* (c). The asterisks on the maps correspond to the shaded clades in (a). These data identify only two well-supported *U. borealis* clades in the mitochondrial DNA (vs. three in the SNP data set), and these clades overlap geographically (b). Two mitochondrial clades are identified in *U. crassa*, but these clades overlap geographically (c) and are differently distributed from populations in the SNP data set

were either previously published GenBank data or are newly generated sequence data (Appendix S1). New DNA extraction and mitochondrial sequence data were generated following protocols in Catullo and Keogh (2014), and quality controlled and assembled in GENEIOUS 11.1.2 (Biomatters). Through the Barcode of Life project (Ratnasingham & Hebert, 2007), we also had sequences for 124 individuals for the cytochrome *c* oxidase I (*COI*) mitochondrial gene for tissues preserved at the Western Australian Museum. Only 70 of these sequences were from the same individuals as the 16S/*ND2* mitochondrial genes, we analysed the *COI* sequences independently, and used the individuals in both data sets to visually confirm congruent phylogenetic topologies between the 16S/*ND2* and *COI* analyses.

Mitochondrial phylogenies were generated using ML and Bayesian coalescent approaches. ML analyses were conducted in IQ-TREE 1.5.6 (Nguyen et al., 2015), with nucleotide substitution model selection using MODELFINDER PRO (Kalyaanamoorthy

et al., 2017). The alignment was partitioned into 16S, and the three codon positions of *ND2* for that analysis, and the three codon positions for *COI* in that analysis. The analyses were run for 10,000 replicates of the ultrafast bootstrap (Minh et al., 2013) and the SH-aLRT (Guindon et al., 2010). Bayesian coalescent analyses were conducted in BEAST 2 version 2.5 (Bouckaert et al., 2014). Models were selected from the MODELFINDER PRO analysis within the IQ-TREE run. Analyses were run in triplicate, using the same partitions as the IQ-TREE analysis, for 20 million generations each, with 10% of the run discarded as burn-in. Convergence of runs and between runs was assessed as per the SNAPP analyses above.

2.7 | Population structure

To identify the number of probable species and interspecific hybridization, we first analysed the filtered SNP data set that included all *U. borealis* and *U. crassa* individuals. Two Bayesian-inferential programs, STRUCTURE 2.3.4 (Pritchard et al., 2000) and NEWHYBRIDS 1.1 (Anderson & Thompson, 2002), were used to infer genetic structuring and assess hybridization across the complex. To determine genetic clusters (K = populations), we evaluated models of K between 1 and 8, with 10 iterations per K. Analyses were run using admixture ancestry and independent allele frequency parameters, with a burn-in of 50,000 and sampling for 100,000 generations. STRUCTURE HARVESTER 0.6.94 (Earl & VonHoldt, 2012) provided an estimation of the best K for all STRUCTURE outputs, determined by the modal ΔK value detailed in Evanno et al. (2005) and combined individual coefficient values between all replicates per K. STRUCTURE results were averaged across all runs. Individuals with a cluster $q \geq .90$ were assessed as having no contemporary admixture and belonging to one of two parental populations. Individuals scoring $q < .90$ were considered hybrids.

We used NEWHYBRIDS (Anderson & Thompson, 2002) to identify the genotype class of individuals identified as admixed in the STRUCTURE analysis. The method assumes that samples are composed of two pure parental populations plus hybrids, where q describes the posterior probability of an individual belonging to a discrete genotype class: either one of two parental classes, F_1 or F_2 hybrids, and their backcrosses. Due to computational limitations of NEWHYBRIDS, we used the gl.nhybrids function in the DARTR 1.8.3 package (Gruber et al., 2018) to reduce to 186 SNPs. Samples were assigned as parental based on the two major groups identified in STRUCTURE analyses, and the function selects loci with fixed differences between the parental clades. NEWHYBRIDS was run on these SNPs with 10,000 burn-in cycles and 50,000 Markov chain Monte Carlo (MCMC) sweeps. All identified F_1 hybrids had a *U. borealis* mitochondrial haplotype (discussed below). We therefore used a two-sided binomial test to assess whether hybrid parentage was sex-biased (i.e., to reject the null hypothesis that the probability of having a *U. borealis* as the maternal parent was equal to .5).

Following the hierarchical approach to STRUCTURE analysis suggested by Janes et al. (2017), we also conducted STRUCTURE analyses on the two major clades identified in the above analyses (*U. borealis* and *U. crassa*). SNPs were independently called and filtered following Figure S2, and excluded interspecific hybrids identified above. These STRUCTURE analyses tested $K = 1{:}5$, and followed all other parameters in the previous analysis.

2.8 | Demographic analyses and expansion tests

To investigate whether the current sympatry of *U. borealis* and *U. crassa* is secondary contact, we assessed the probable origin of *U. crassa* (i.e., Kimberley vs. Top End). In particular, we wanted to test whether our genetic data were consistent with *U. crassa* having expanded from the Top End into the Kimberley where it is now sympatric with *U. borealis*. As a species' range expands, genetic drift associated with colonization results in reductions in genetic diversity and heterozygosity at the leading edge of the range expansion (Austerlitz et al., 1997; Ramachandran et al., 2005). Accordingly, if *U. crassa* has undergone a range expansion, it should have higher genetic diversity and heterozygosity in the ancestral region. We therefore assessed range-wide patterns of genetic diversity in *U. crassa* by calculating average expected heterozygosity (DARTR; Gruber et al., 2018) and Shannon's Information Index (GENALEX; Peakall & Smouse, 2006) for the *U. crassa* populations identified by the STRUCTURE analysis. We tested whether there were significant differences in expected heterozygosity between populations of *U. crassa* using the gl.test.heterozygosity function in the DARTR package, which uses a rerandomization approach to assess pairwise differences between populations. We used the gl.report.heterozygosity function in the DARTR package to calculate the observed heterozygosity for all *U. crassa* individuals, and used a Wilcoxon rank sum exact test to test for differences in observed heterozygosity between the two geographical regions.

We tested both *U. crassa* populations for mutation–drift equilibrium by calculating Tajima's D (Tajima, 1989). Significant negative values of Tajima's D are due to an excess of rare alleles and are consistent with range expansion. Significant positive values are associated with population contraction. We tested whether Tajima's D was significantly different from zero by creating coalescent simulations of Tajima's D in MS (Hudson, 2002) and generating a two-tailed p-value for each population (Hahn, 2018). We also tested for spatial expansion using the approach outlined in Peter and Slatkin (2015), which applies a founder effect algorithm to test for population expansion and estimate the source of that expansion. This method infers the most likely expansion of origin by assuming populations expand on a stepping stone model from a single population in the past.

2.9 | Acoustic signals

Recordings of frog calls from across the species complex were sourced from the Western Australian Museum and recorded in the field, primarily by the authors (PD and RAC; Appendix S1).

TABLE 1 Summary of temperature-corrected advertisement call characteristics for each species overall, allopatric (top end) and sympatric (Kimberley) populations of *Uperoleia crassa*, and data for the subsets of Kimberley *U. crassa* with and without a characteristic final pulse (see Figure S3)

Species	Group	*n*	Pulse number (pulses [*SD*])	Pulse rate (pulses s^{-1} [*SD*])	Call suration (s [*SD*])	Proportion of individuals with final pulse
U. borealis	All	18	20.27 [2.91]	110.65 [13.60]	0.178 [0.015]	0
U. crassa	Top End (allopatric) - all	20	10.46 [1.50]	72.01 [9.25]	0.148 [0.017]	0.030
	Kimberley (sympatric) - all	18	8.05 [1.70]	49.22 [13.83]	0.171 [0.016]	0.833
	With final pulse	15	6.82 [0.91]	37.60 [5.69]	0.182 [0.014]	1
	Without final pulse	3	11.60 [1.02]	81.40 [3.62]	0.143 [0.016]	0
Hybrid	Up0165		14.33	69.02	0.21	2 of 3 calls
	Up0176 call 1		12.33	94.61	0.13	0 of 3 calls
	Up0176 call 2		9.67	127.78	0.08	0 of 3 calls

Notes: For all individuals, we measured three calls per individual and averaged values across calls. Also presented are raw (not temperature-corrected) data describing the calls of hybrid individuals. Hybrids ($n = 2$) are identified by their laboratory ID numbers. Hybrid Up0176 produced two distinct advertisement calls in a single recording (in addition to an alert call). Both hybrid individuals have a *Uperoleia* mitochondrial haplotype, and hybrid Up0165 is an F_1 hybrid (nuclear DNA analysis; see text for details). Columns are the number of individuals for each group (*n*), the average number of pulses produced, the average call duration (s), the average pulse rate (pulses s^{-1}) and the proportion of individuals that produced at least one call with the characteristic final pulse (for hybrids, we present the number of calls with the final pulse out of three).

Most recordings were made on a Marantz solid state recorder with Samson Q7 microphones. Variations in recording equipment are not likely to impact the temporal characteristics reported here. Species within this complex produce two distinct acoustic signals with different hypothesized functions. The first appears to be an advertisement call produced in active choruses and consists of a "squelching" sound with evenly spaced pulses (Figure S3a,b). The second call type is a faster, "sharper" sound (Figure S3c), which often has structural components (e.g., an internal pause and decreasing interpulse intervals over the duration of the call). This second call type was observed primarily when the chorus was disturbed or, in some cases, after the same call type was produced by a neighbouring frog (PD & RAC, personal observation; Robertson, 1984, 1986). We hypothesize this second call functions as an alert or aggression call. We restrict all further discussion of calls to the advertisement calls.

Advertisement call data were available for 58 males (18 *U. borealis*, 38 *U. crassa* and two hybrid individuals). Of these individuals, we had SNP data for four *U. borealis*, six Kimberley *U. crassa* and one Top End *U. crassa*. We had mitochondrial sequence data for 12 *U. borealis*, 13 Kimberley *U. crassa* and two Top End *U. crassa*. Individuals without sequence data were assigned to species based on the similarity of their calls (based on principal component scores) to those of sequenced individuals, as well as their geographical location. One individual we identified as a hybrid based on its unique advertisement call, which differed from both parental species, did not have SNP data, but had a *U. borealis* mitochondrial haplotype.

Calls were measured using RAVEN PRO 1.6.1 (The Cornell Laboratory of Ornithology). Each individual recording was assessed to determine whether the individual produced an advertisement call, alert call or both call types. Downstream analyses were carried out only on the advertisement calls because advertisement calls are the primary means by which receivers identify appropriate mates in acoustically communicating frogs, and are thus linked to reproductive isolation of diverging species (Gerhardt & Huber, 2002; Wells, 2010).

We measured the following call characteristics for three calls produced by each male: pulse number, pulse rate (pulses per second [pps]), call duration (seconds), and the presence or absence of the characteristic "final pulse" whose preceding interpulse interval was shorter than the interpulse intervals in the rest of the call (Figure S3b). We scored the presence or absence of the final pulse (1 if present, 0 if absent) visually using each recording's waveform. Measured trait values vary continuously for pulse number, pulse rate and call duration. Some males showed within-individual variation in final pulse production, producing calls both with and without the characteristic final pulse; thus, possible scores for the "final pulse" variable were 0, 0.333, 0.667 or 1.

Because temporal properties of anuran acoustic signals are temperature-dependent, we first temperature-corrected the pulse number, pulse rate and call duration data to 28°C following the methods of Platz and Forester (1988). Call characteristics were summarized for the following subsets of *U. crassa*: (i) only the Top End ("allopatric") individuals, (ii) only the Kimberley ("sympatric") individuals, and the Kimberley individuals with (iii) and without (iv) the final pulse (Table 1). Hybrid individuals were not included in any downstream call analysis. Because there were not enough recordings of hybrid individuals to identify the relationships between temperature and call characteristics, their calls are described separately and without temperature correction in Table 1. The RCD hypothesis predicts that signal characteristics relevant to species identification should be more different in sympatric populations of two different species than in allopatric populations of either. To test this prediction, we used analysis of variance (ANOVA) with Tukey's range test to compare the call characteristics of *U. crassa* populations sympatric with *U. borealis* to those allopatric with *U. borealis*.

To assess the clustering of individuals' advertisement calls relative to their species identity and geographical region, we

first conducted a principal components analysis (PCA), using the *prcomp* function in the *stats* R package (R Core Team, 2021). Call variables were scaled to have unit variance prior to analysis. We fit two separate generalized linear models to test for differences among species groups in each of the first two principal component scores.

Finally, we used a *k*-means clustering analysis (Hartigan & Wong, 1979) to assign *U. crassa* individuals to groups on the basis of call characters alone. If RCD is driving variation in call characters, we would expect naïve clustering to produce groups that reflect the presence or absence of *U. borealis*. The *k*-means analysis was carried out using the *kmeans* function in the base R package "*stats*" (R Core Team, 2021), and clustered samples into $k = 2$ groups based on the same advertisement call characteristics we examined using the PCA. To examine the geographical transition zone between the advertisement calls of sympatric and allopatric *U. crassa*, we used logistic regression to assess whether longitude was a significant predictor of cluster assignment and to find the "transition zone" between sympatric (western) and allopatric (eastern) *U. crassa* call phenotypes.

2.10 | Morphology

Quantitative morphological data were collected for 90 individuals across the complex. All measurements listed below were able to be taken on 30 males and 10 females for genotyped *U. borealis*, 23 males and one female for genotyped *U. crassa*, and two genotyped F_1 hybrid males as per the NEWHYBRIDS analysis results (Appendix S1). An additional 14 *U. crassa* individuals from the Top End without genotyping were also measured. These data follow previous taxonomic revisions (Catullo, Doughty, et al., 2014), and included snout–urostyle length (SUL), eye–naris distance (EN—from anterior corner of the eye to the midpoint of the closest nostril), interorbital distance (IO—from anterior corners), internarial distance (IN—from medial margins of nares), eye length (EyeL—from corner of anterior and posterior edges), arm length (ArmL—from elbow to tip of 3rd finger), tibia length (TL) and foot+tarsus length (FTL—from knee to tip of 4th toe). All measurements were taken with electronic callipers to the nearest 0.1 mm. Variation in morphology was analysed using a PCA, using the prcomp function in the *stats* R package (R Core Team, 2021). Morphological data were scaled to have unit variance prior to analysis.

3 | RESULTS

3.1 | Nuclear DNA sequencing and SNP filtering

Over 100,000 polymorphic SNP loci were scored in each of the data sets prior to filtering with strict quality parameters. The overall number of unfiltered SNPs, average loci call rate, average reproducibility, average read depth and number of SNPs retained after stringent filtering are shown in Figure S2. The total number of SNPs retained varied from 1938 (ML phylogeny) to 14,032 (*Uperoleia crassa* population structure) across the data sets (Figure S2).

3.2 | Phylogenetic analysis of SNP data

In the individual-level ML analysis of the SNP data, the monophyly of both *Uperoleia borealis* and *U. crassa* species was highly supported (Shimodaira-Hasegawa test (SH) = 100, ultrafast bootstrap (UFboot) = 100; Figure 1a). *U. borealis* individuals form three distinct, highly supported clades (SH = 100, UFboot = 100), which align with the populations identified in the STRUCTURE analysis (see below). *Uperoleia crassa* individuals form an extensive comb, with the Kimberley clade (Pop. 4) forming a single, well-supported clade within the overall species (SH = 100, UFboot = 100). This analysis found low support for the sister species status of *U. borealis* and *U. crassa* (SH = 82.5, UFboot = 99; Figure 1a). Instead, both species form a soft polytomy with other *Uperoleia* that share similar advertisement calls and morphology (Catullo & Keogh, 2014).

The species tree analyses gave broadly similar results to the individual-level phylogeny (Figure 1b). The SNAPP analysis found strong support for the monophyly of *U. borealis* (Posterior probability (PP) = 1) but was not able to resolve the relationship between the three geographical populations identified in the STRUCTURE analysis. Both populations of *U. crassa* were strongly supported as a monophyletic clade (PP = 1). Relationships between each of these species and *U. talpa* and the *U. saxatilis*/*U. russelli* clade were poorly supported.

3.3 | Mitochondrial phylogeny

The mitochondrial phylogeny from the genes 16S and *ND2* (Figure 2a) recovered the same structure as the phylogeny generated using *COI* (Figure S5), and the SNP-based phylogeny (Figure 1a). Broadly, the mitochondrial phylogenies are unable to resolve relationships between the five species in this clade. Within our target taxa, all *U. borealis* individuals form a well-supported clade (SH = 96.3, UFboot = 99, PP = 1; Figure 2a). All six *U. borealis*/*U. crassa* F_1 hybrid individuals (as identified in the population genetic analysis; Figure 3a) had *U. borealis* mitochondria, indicating they had *U. borealis* mothers. This proportion was significantly different than expected by chance (two-sided binomial test, H_O: $\Theta = 0.5$; 95% CI: [0.541, 1.000], $p = .03$), suggesting that hybridization may be unidirectional.

Within *U. borealis*, there are two well-supported mitochondrial clades, broadly corresponding to the east and west Kimberley, with overlap in the southern Kimberley. These clades overlap in the region occupied by *U. borealis* Pop. 2 in the STRUCTURE analysis (Figure 3b). Mitochondrial data also support the monophyly of *U. crassa* (SH = 100, UFboot = 100, PP = 1). Mitochondrial data split *U.*

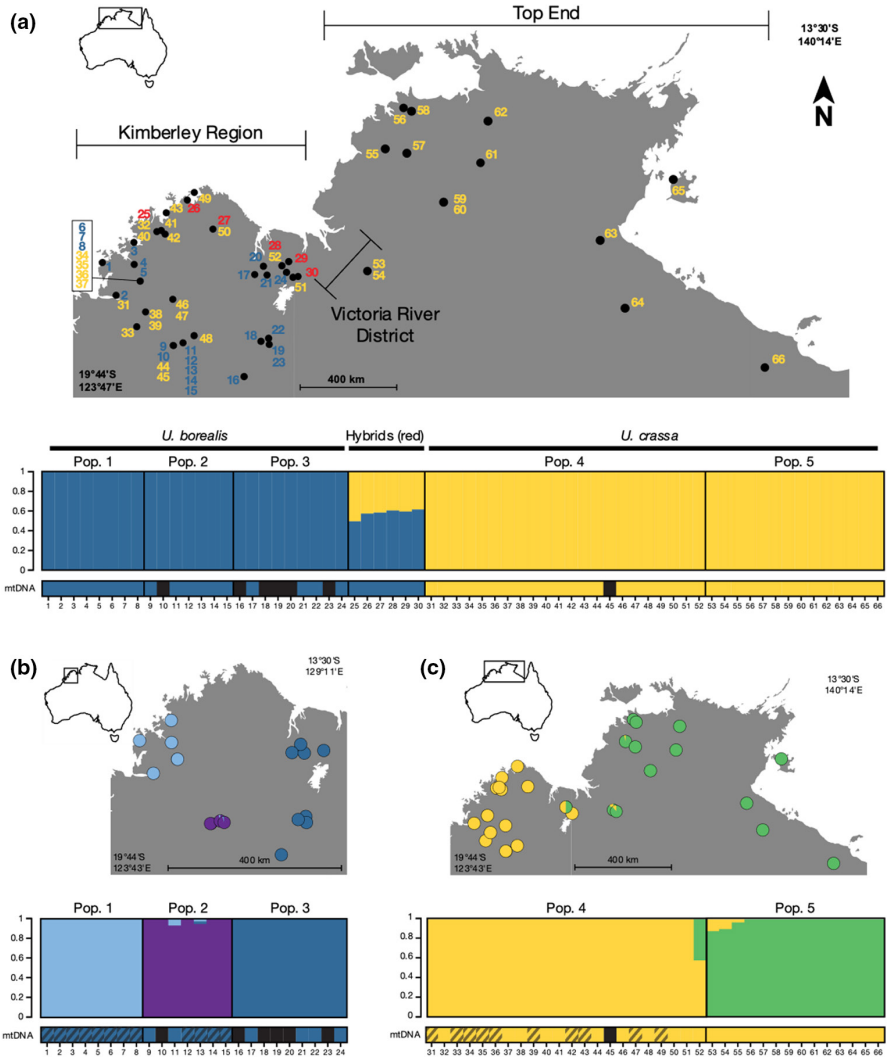

FIGURE 3 Distribution maps, STRUCTURE results and mitochondrial lineage assignments of *Uperoleia borealis* and *Uperoleia crassa* samples. The "Top End," "Kimberley" and "Victoria River District" are identified as referenced in the main text. In the map in (a), *U. borealis* is presented in blue numbers, *U. crassa* is presented in yellow numbers and F_1 hybrids are presented in red numbers. Map numbers identify individuals in the STRUCTURE result below. The barchart in (a) is the result of the Bayesian clustering of individuals from the program STRUCTURE, which is based on allele frequencies of the nuclear DNA. Below this is the colour-coded mitochondrial DNA clade of each individual at the species level (see Figure 2; black represents no mitochondrial sequence data). In (a), all hybrid individuals have a *U. borealis* mitochondrial DNA haplotype. Separate STRUCTURE analyses are shown for *U. borealis* (b) and *U. crassa* (c). For each of these species, the within-species mitochondrial lineage distributions differ substantially from the structure identified by the nuclear DNA

crassa into a restricted northwest Kimberley clade, and a widespread mitochondrial clade that covers the remaining ~1000 km of the distribution (Figure 2c).

3.4 | Population genetic analyses of SNP data

The initial STRUCTURE analysis (Figure 3a) investigating admixture between *U. borealis* and *U. crassa* identified $K = 2$ as the optimal clustering. The first cluster represents *U. borealis* and is restricted to the Kimberley region. The second cluster is widespread and includes all individuals of *U. crassa*, both sympatric (Kimberley) and allopatric (Top End). Across the northern Kimberley, six individuals were identified as admixed ($q < .9$), with maximum population ancestry coefficient values of $.503 < q < .618$ in these individuals (Figure 3a, red). The PCoA (Figure S4) exhibited clear differentiation between the same two groups, divided on PC1 (52.7% of the variance). PC2 accounted for 13.3% of the variance, and divided *U. borealis* into three distinct geographical clusters. The same admixed individuals in the STRUCTURE analysis were intermediate in the PCoA (Figure S4, green). NEWHYBRIDS assigned all six admixed individuals as F_1 hybrids (Table S1; posterior probability of 1 for all hybrid individuals).

Further STRUCTURE runs were conducted to infer the intraspecific population structure within respective *U. borealis* and *U. crassa* species groups, for which STRUCTURE HARVESTER identified $K = 3$ and $K = 2$, respectively, as the optimal clustering. *U. borealis* individuals were assigned to three populations (Figure 3b; Pop. 1, Pop. 2, Pop. 3). Population 1 ($n = 7$) of *U. borealis* is restricted to the northwestern Kimberley Plateau and Pop. 3 ($n = 9$) is present in the lowland east Kimberly. Population 2 ($n = 8$) is located in the southern Kimberley Plateau, and exhibits some mixing between Pop. 1 and Pop. 3, but are not identified as admixed as q was greater than .9 for all individuals. *Uperoleia crassa* individuals were assigned to two overlapping populations (Figure 3c; Pop. 4 and Pop. 5). Population 4 ($n = 22$) is distributed across the entire Kimberley region. Population 5 ($n = 14$) spans the region from the eastern Kimberley to the Gulf of Carpentaria near the Northern Territories–Queensland border. The STRUCTURE analysis of *U. crassa* (Figure 3c) identified admixture between Pop. 4 and Pop. 5 through the Victoria River district.

3.5 | Population genetics and evidence for species' geographical origin

The expected heterozygosity was significantly lower ($p < .001$) in the sympatric (Kimberley) population of *U. crassa* (Pop. 4; $H_E = 0.063$) compared to the allopatric (Top End) population (Pop. 5; $H_E = 0.134$), despite sample size differences ($N = 22$ vs 14, respectively). The Wilcoxon rank sum exact test on individual observed heterozygosities found a positive, statistically significant, and very large effect of geographical region, with the allopatric (Top End) population significantly higher (Figure 4; $W = 302.00$, $p < .001$; r (rank biserial) = 0.96, 95% CI [0.93, 1.00]). The pattern of genetic diversity as measured by heterozygosity was reflected in Shannon's Information Index, where sympatric (Kimberley) *U. crassa* showed lower diversity ($I = 0.237$, $SE = 0.003$) than allopatric (Top End) *U. crassa* ($I = 0.400$, $SE = 0.003$). Tajima's *D* differed between the two populations, with a more negative and barely significant value in the sympatric population (−1.4622; $p = .046$) vs. the nonsignificant allopatric population result (−1.0251; $p = .164$). The range expansion analysis using the Peter and Slatkin, (2015) model identified the origin of *U. crassa* as in the Top End (Figure 4).

3.6 | Advertisement calls

Pulse characteristics, including pulse number, pulse rate and the presence of a final pulse, differed significantly among groups (ANOVA pulse number: $F_{(2,53)} = 171$, $p < .001$; pulse rate: $F_{(2,53)} = 114.9$, $p < .001$; final pulse: $F_{(2,53)} = 68.56$, $p < .001$; Figure 5f–h). On average, sympatric *U. crassa* had fewer pulses and a slower pulse rate than allopatric *U. crassa*, resulting in a longer call duration (Table 1). Sympatric *U. crassa* were also very likely to have a characteristic final pulse relative to the other two groups (83.3% of individuals compared to 3% of allopatric *U. crassa* and 0% of *U. borealis*; compare Figure 5c to 5a,b). Allopatric *U. crassa* differed from both sympatric *U. crassa* and *U. borealis* in call duration (ANOVA call duration: $F_{(2,53)} = 18.31$, $p < .001$; Figure 5h). Descriptive statistics for the temperature-corrected advertisement calls are presented in Table 1; complete output from Tukey's range tests comparing groups is given in Table S2.

We conducted a PCA to reduce the dimensionality of the call characteristics (Figure 5i). The first two principal components explained 94.6% of the variance in advertisement call traits (PC1: 63.8%; PC2: 30.8%; Table 2). PC1 was heavily loaded by pulse characteristics (a combination of the presence or absence of the final pulse, pulse number and pulse rate) while PC2 was predominately loaded by call duration (Table 2). To determine whether *U. crassa* call characteristics differed from *U. borealis* more in sympatry than in allopatry (Figure 6a), we fit two generalized linear models to separately examine how the first two principal components differed by species group. Consistent with the predictions of the RCD hypothesis, call characteristics of *U. borealis* and *U. crassa* differed more in sympatry than in allopatry, and these differences were largely driven by pulse characteristics. Sympatric *U. crassa* differed significantly from *U. borealis* in PC1, which was heavily loaded by pulse characteristics ($\beta = 3.610$, $t = 16.89$, $p < .001$; Figure 6b). Allopatric *U. crassa* also differed from *U. borealis* in PC1 ($\beta = 2.133$, $t = 10.24$, $p < .001$; Figure 6b) but to a lesser extent. PC2, which was heavily loaded by call duration, showed a different pattern (Figure 6c): allopatric *U. crassa* differed from *U. borealis* in call duration ($\beta = -1.554$, $t = -6.016$, $p < .001$), while sympatric *U. crassa* did not ($\beta = 0.150$, $t = 0.566$, $p = .574$).

To determine whether *U. crassa* individuals could be naively assigned to their geographical regions based on multivariate variation in their call characteristics, we performed a *k*-means clustering analysis and specified two groups. The algorithm split *U. crassa* ($n = 38$)

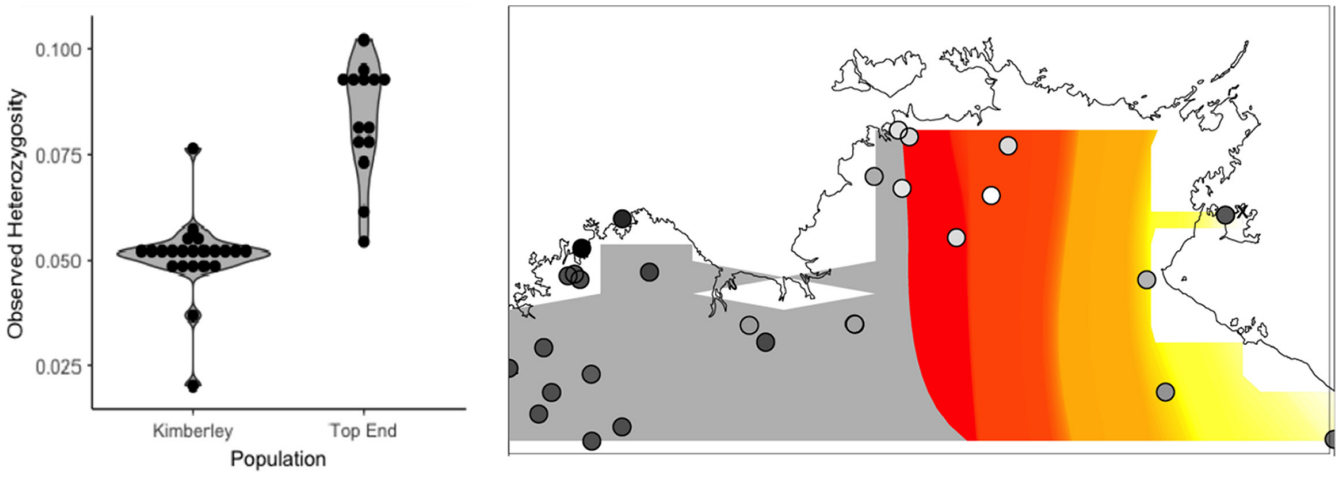

FIGURE 4 Geographical patterns of heterozygosity and range expansion in *Uperoleia crassa*. The observed heterozygosity values for individuals from the Kimberley and top end populations are shown as violin plots (left) and were significantly different ($p < .001$). The results of the RANGEEXPANSION analysis (right), which identify the probable origin of expansion, show the distribution of individuals and their relative heterozygosity (dark circle is low, light circle is high). The probable expansion origin, as indicated by yellow colour and the X, is identified as the eastern top end

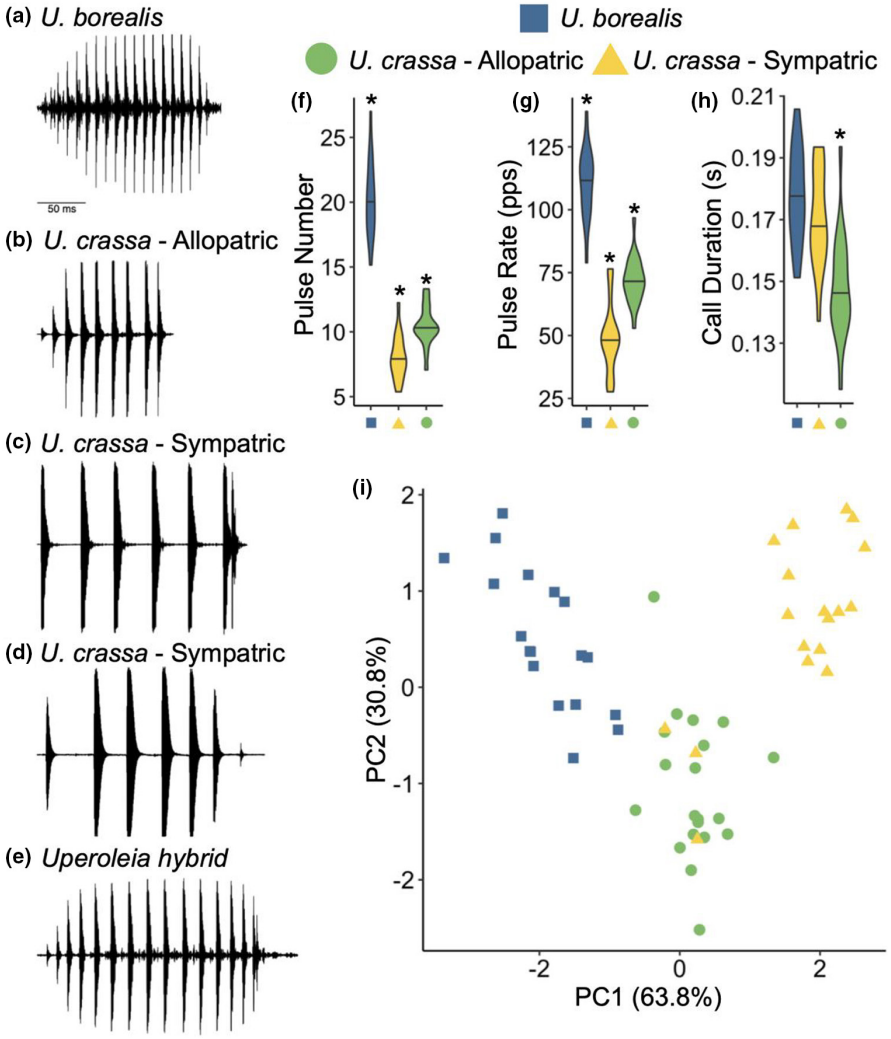

FIGURE 5 Variation in advertisement signals within the species complex. Waveforms are shown in (a)–(e); the scale bar in (a) applies to all waveforms. (b–d) Variation in *Uperoleia crassa* signals, which differ between allopatric (b) and sympatric (c and d) populations in the top end and Kimberley, respectively. Within sympatric populations, some individuals produce calls with a characteristic "final pulse" while other calls lack such a final pulse (compare panels c and d). (e) the advertisement call of a male confirmed to be a *U. borealis*/*U. crassa* hybrid using nuclear DNA. Violin plots show variation among groups (blue squares: *U. borealis*; yellow triangles: Sympatric *U. crassa*, green circles: Allopatric *U. crassa*) in pulse number (f), pulse rate (g) and call duration (h), with horizontal black bars showing group means. Star notation in (f)–(h) denotes when the starred group is statistically different from both other groups based on Tukey's range tests in Table S2. (i) the first two principal components from the PCA cumulatively explained 94.63% of the variation in temperature-corrected advertisement call traits. Advertisement call data generally supported the prediction that *U. crassa* and *U. borealis* calls should differ more from each other in sympatry than in allopatry, and these differences were mainly driven by pulse characteristics

into two clusters of sizes 23 and 15 (Figure S7). Cluster assignments corresponded closely to the individuals' true geographical locations, with only three individuals of the "western" (sympatric) call type "misassigned" to the cluster representing the "eastern" (allopatric) call type (Figures 6a,d and S6). We fit a logistic regression model to examine how cluster assignment was related to geographical location and found that longitude was a significant predictor of cluster assignment ($\beta = 1.275$, $t = 2.91$, $p = .0036$) and that cluster assignment switched from the western to the eastern cluster at 127.65° of longitude (Figure 6d).

3.7 | Morphology

Uperoleia borealis and both populations of *U. crassa* were indistinguishable in our morphological analysis. That is, the groups overlapped across all morphological measurements examined (Table S3). Principal components scores did not group into any distinct clusters with genetic clades identified using nuclear or mitochondrial data (Figure S8). We did confirm, however, that *U. borealis* possesses more extensive webbing between the toes (not included in the meristic analysis) than *U. crassa*.

TABLE 2 Matrix of variable loadings and importance of components from the principal components analysis (PCA) examining variation in call traits

Trait	PC1	PC2	PC3	PC4
Final pulse	0.495825	0.462776	0.734780	0.009704
Pulse number	−0.600269	0.215784	0.278635	−0.717967
Pulse rate	−0.612128	−0.030926	0.423730	0.666930
Call duration (s)	−0.138327	0.859255	−0.450460	0.199080
SD	1.5978	1.1101	0.44815	0.1174
Proportion of variance	0.6383	0.3081	0.05021	0.0035
Cumulative proportion of variance	0.6383	0.9463	0.99655	1.0000

Notes: The original traits included in the PCA include the presence or absence of the final pulse (0 or 1), pulse number, pulse rate (pulses s^{-1}) and call duration (s). The last three rows of each column show the standard deviation, proportion of variance explained and cumulative portion of variance explained by each principal component.

4 | DISCUSSION

4.1 | Species delimitation

Both the nuclear SNP data and mitochondrial data support two species, corresponding to *Uperoleia borealis* in the Kimberley region, and the combined *Uperoleia crassa* and *Uperoleia inundata* individuals from the Kimberley and Top End (hereafter *U. crassa*). Despite the presence of hybrid individuals, we found no evidence that hybrids backcross with parental *U. borealis* or *U. crassa*: all six hybrids we detected were identified as F_1 hybrids with the *U. borealis* mitochondrial haplotype and there was no evidence of further introgression.

The two genetic clades had distinct male advertisement calls but were morphologically indistinguishable in our analyses. These differences in advertisement calls, and subtle differences in webbing and other variable characters, are why *U. crassa* and *U. inundata* were originally described as distinct species (Tyler et al., 1980). Because advertisement calls are the primary means by which female frogs discriminate among potential mates, changes in advertisement calls are expected to further drive reproductive isolation between frog species (Gerhardt & Huber, 2002; Wells, 2010). We found that the call of *U. crassa* varies geographically, but on average features approximately half as many pulses as *U. borealis* produced at a slower pulse rate, resulting in a similar call duration. Our combined acoustic and genetic data support the presence of only two post-zygotically isolated species within this group. These species are *U. borealis* and *U. crassa*. We here synonymize *U. inundata* into *U. crassa* (Tyler et al., 1980).

In the next section, we discuss the genetic and acoustic evidence that a westward-invading population of *U. crassa* into the Kimberley from the Top End resulted in reproductive interference from the Kimberley-endemic *U. borealis*.

4.2 | Population history and range expansion

Two sequential landscape-scale processes underlie the origin of RCD; divergence, probably in allopatry, followed by secondary contact through range expansion (Cooley et al., 2006; Geyer & Palumbi, 2003; Higgie et al., 2000; Kulathinal & Singh, 2000; Waage, 1979). The Top End and Kimberley represent separate areas of species and phylogeographical endemism (Moritz et al., 2016; Rosauer et al., 2009), separated by the Victoria River region, which is variously considered as a transition zone or barrier (Catullo, Doughty, et al., 2014; Melville et al., 2011; Potter et al., 2012). If *U. crassa* arose in the Top End and *U. borealis* in the Kimberley, the current sympatry in the Kimberley would be secondary, resulting from the east to west expansion of *U. crassa* into the range of *U. borealis*.

Our data are broadly supportive of this hypothesis. The divergence between the two species has been previously dated to 1.8 million years ago [0.74–3.07] (Catullo & Keogh, 2014). This date corresponds to the beginning of the Pleistocene, a period of shifts from woodlands to grasslands in the inland tropical areas (Martin, 2006), which would have separated woodlands in the Top End from those in the Kimberley. The distribution of genetic diversity in *U. crassa* is consistent with a Top End origin and westward expansion into the Kimberley. We found significantly higher genetic diversity in the Top End population of *U. crassa* as measured by the Shannon Information Index, significantly higher observed heterozygosity in the Top End, and heterozygosity-based methods identify the range expansion origin as the far east of the Top End (Peter & Slatkin, 2015). Expansion statistics that depend on rare alleles (Tajima's *D*; Tajima, 1989) identified a signature of expansion in the Kimberley but not the Top End.

The consequences of this biogeographical scenario are important to the patterns of RCD. In the Kimberley, the invading *U. crassa* females would have needed to distinguish conspecific males among a much larger number of heterospecific *U. borealis* males (Contarini et al., 2009; Kishi, 2015; Kyogoku & Nishida, 2012). Given the lack of conspicuous morphological differences between the species, there are two outcomes for sympatric *U. crassa*: undergo reproductive character displacement (i.e., call divergence) or population extinction (reviewed in Kyogoku & Wheatcroft, 2020).

4.3 | Reproductive character displacement

The RCD hypothesis makes predictions about population-level traits and about hybrid fitness. At the population level, if invading *U. crassa* females mismated with *U. borealis* males, there should be strong local

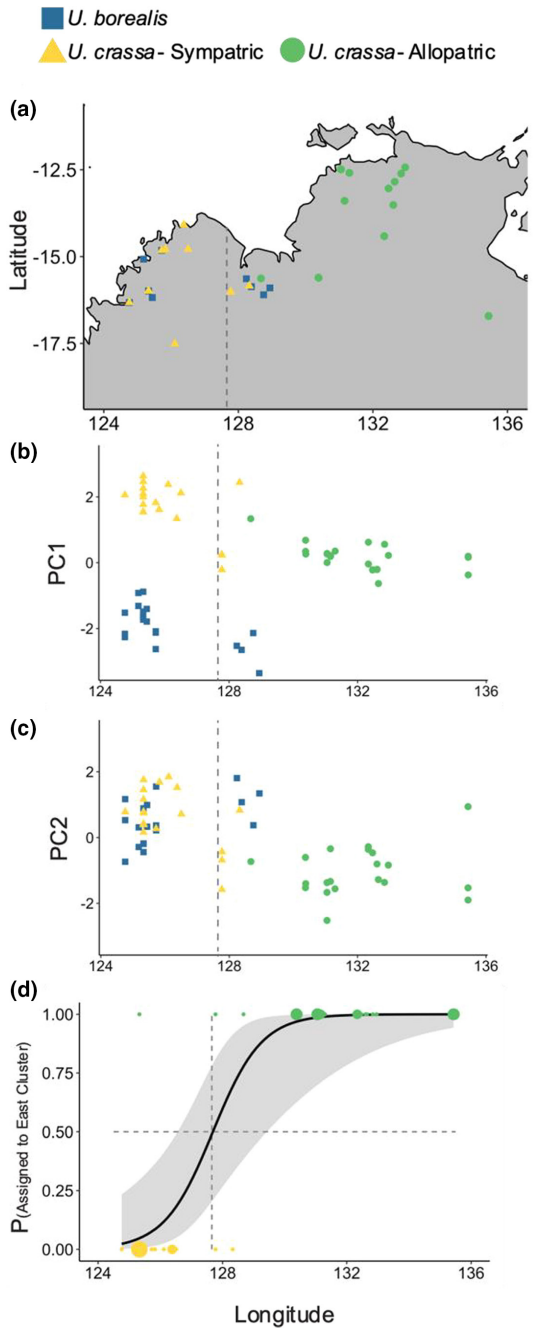

FIGURE 6 Geographical variation in advertisement call characteristics supports the reproductive character displacement hypothesis. (a) Map of advertisement call recording locations by species group. (b) PC1 (loaded largely by pulse characteristics) in relation to longitude. *Uperoleia crassa* and *U. borealis* differ more in sympatry than in allopatry. (c) PC2 (loaded predominately by call duration) in relation to longitude. (d) *K*-means cluster analysis naïvely assigned *U. crassa* individuals to broad "western" (yellow) and "eastern" (green) clusters based on their advertisement call characteristics. The geographical "transition zone" at which frogs were more likely to be assigned to the eastern cluster than expected by chance was 127.65 degrees of longitude. Key at top of figure applies to (a)–(c)

selection for the advertisement calls of *U. crassa* to diverge from *U. borealis* in sympatry more than in allopatry. Consistent with this prediction, we found that advertisement calls of *U. crassa* and *U. borealis* differed more in the Kimberley, where both occur, than in the Top End, where *U. crassa* occurs alone. These differences were largely driven by pulse characteristics, with *U. crassa* calls shifting in sympatry toward fewer pulses at slower pulse rates, and acquiring the characteristic final pulse that was nearly absent from the allopatric populations. Call duration, which is necessarily related to changes in pulse number and rate, did not show the same pattern. In many anuran species, pulse characteristics are important signals of species recognition and have relatively frequently been documented as examples of traits undergoing reproductive character displacement (Fouquette, 1975; Lemmon, 2009; Littlejohn, 1965; Loftus-Hills & Littlejohn, 1971; Schul & Bush, 2002). Pulse characteristics often show little variation within individuals and thus tend to be suitable for reliably discriminating between conspecific and heterospecific males (Gerhardt, 1991; Shaw & Herlihy, 2000; Tanner & Bee, 2020). By contrast, call duration is often considered a signal of mate quality because of the intrinsic positive relationship between call duration and energy expenditure (Welch et al., 1998; Wells & Taigen, 1986, 1989). Our data are supportive of these signal functions applying in *Uperoleia*, as we demonstrated RCD in pulse characteristics but not in call duration. Future research should ascertain the function of these signal components and patterns of within-individual variation in signal production.

A necessary condition for reinforcement is the reduction in fitness of hybrids (Schlefer et al., 1986), which results in RCD that allows increased ability for females to identify conspecific males (reviewed in Pfennig, 2016). Hybrids are likely to produce calls with intermediate phenotypes that are less attractive to females of either parental species, and therefore have reduced likelihood of mating (Höbel & Gerhardt, 2003; Tucker & Gerhardt, 2012). If hybrids have reduced fitness due to reduced viability, we should also expect to see few hybrids relative to either parental species. While evidence for reduced hybrid viability is best supported by direct experimentation (Arnold & Hodges, 1995; Lemmon & Lemmon, 2010), our acoustic and genetic data are consistent with reduced hybrid reproductive fitness. Using nuclear DNA analyses we identified one hybrid individual (Up0166) that had an associated call recording. Up0166 had an intermediate pulse number paired with the characteristic final pulse usually associated with sympatric *U. crassa*. The resulting call was noticeably different from the advertisement calls of both parental species. Another suspected hybrid (Up0176; *U. borealis* mitochondrial haplotype) produced two different advertisement call variants (the only individual to do so), neither of which was similar to the parental species (Figure 5e). Another line of evidence supporting reduced hybrid fitness came from the lack of admixture between parental *U. borealis* and *U. crassa*, contrary to what would be expected if hybrids regularly backcrossed to parental species. Given that hybridization is likely to induce changes in advertisement calls that have significant effects on female choice (Höbel & Gerhardt, 2003; Lemmon & Lemmon, 2010), the presence of F_1 hybrids combined with the lack of genetic admixture between parentals suggests that male hybrids suffer from reduced attractiveness to females.

The patterns of hybridization found in the combined nuclear and mitochondrial data are also consistent with the hypothesis that

U. crassa in the Kimberley are under selection for better conspecific recognition. Where an expanding species undergoes strong selection for recognition of conspecifics (here, *U. crassa*), the previously occurring species (here, *U. borealis*) should not prefer conspecifics as strongly owing to a lack of selection driven by mismatings (Hoskin et al., 2005). Our nuclear data identified six F_1 hybrid individuals, all of which carried *U. borealis* mtDNA, indicating that all resulted from crosses of female *U. borealis* and male *U. crassa*. Although hybrid inviability of the opposite cross cannot be ruled out (Wirtz, 1999), this unidirectional hybridization could be due to stronger conspecific selection by *U. crassa* females, consistent with the high costs of mismating with *U. borealis* males (Hsiao et al., 2021). Future research should determine whether the apparent shift in U. crassa advertisement signals documented here (i) improves receiver discrimination between conspecific signals and *U. borealis* signals or (ii) is related to changes in receiver selectivity across the geographical gradient (Gerhardt, 2013; Lemmon, 2009; Littlejohn, 1965). Such data would constitute key evidence that RCD was the driver of the regional differences in signals we measured in *U. crassa*.

Here, we have presented genetic and behavioural evidence supporting the hypothesis that *U. crassa* originated in the Top End, then dispersed westward into the Kimberley, where it faced reproductive interference with the native *U. borealis* that subsequently drove RCD in advertisement signals. Our study illustrates the power of population genomics to generate and test hypotheses about how and why mating signals evolve among species in the presence of a recently evolving sister lineage.

AUTHOR CONTRIBUTIONS

RAC and JSK designed the project. RAC, MRW and PD conducted fieldwork. FJ, RAC, MRW and JCT analysed and interpreted data. All authors contributed to drafting the manuscript, led by RAC and JCT.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

OPEN RESEARCH BADGES

This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at [provided https://doi.org/10.5061/dryad.h18931zpd].

DATA AVAILABILITY AND BENEFIT SHARING

Mitochondrial gene sequences are available on GenBank (details in Appendix S1). Raw sequence data are available on the sequence read archive (PRJNA843732). Unfiltered SNP data, morphological and acoustic measurements, and R code for SNP filtering and acoustic analyses are available on Dryad (10.5061/dryad.h18931zpd).

ORCID

Frederick R. Jaya https://orcid.org/0000-0002-4019-7026
Renee A. Catullo https://orcid.org/0000-0002-1790-7085

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