Zootaxa 4858 (2): 201–230
https://www.mapress.com/j/zt/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.4858.2.3
http://zoobank.org/urn:lsid:zoobank.org:pub:82613E60-CEEB-4BCB-8536-007CD888606E

A new species of frog in the *Litoria ewingii* species group (Anura: Pelodryadidae) from south-eastern Australia

MICHAEL MAHONY[1*], BEDE MOSES[1,5], STEPHEN V. MAHONY[2,6],
FRANK L. LEMCKERT[2,3] & STEPHEN DONNELLAN[4]
[1]*School of Environmental and Life Sciences, The University of Newcastle, University Drive, Callaghan, 2308, Australia.*
[2]*Australian Museum Research Institute, Australian Museum, 1 William St, Sydney 2010, Australia.*
[3]*Current address: Eco Logical Australia Pty Ltd, PO Box 12, Sutherland, 2232, Australia.*
Frank.Lemckert@ecoaus.com.au; https://orcid.org/0000-0002-3844-6185
[4]*South Australian Museum, North Terrace, Adelaide, Adelaide, 5000, Australia.*
Steve.donnellan@samuseum.sa.gov.au; https://orcid.org/0000-0002-5448-3226
[5] *bedemoses@gmail.com; https://orcid.org/0000-0002-1736-0075*
[6] *Stephen.mahony93@gmail.com; https://orcid.org/0000-0002-2379-0952*
[*]*Corresponding author.* *michael.mahony@newcastle.edu.au; https://orcid.org/0000-0002-1042-0848*

Abstract

Population declines and range contractions among Australian frogs that commenced in the early 1980s continue in some species that were once widespread. The generality of this pattern has been difficult to discern, especially for those species that are encountered rarely because they have restricted periods of calling activity with poorly defined habitat preferences, and are not common. Several lines of evidence indicate that *Litoria littlejohni* is such a species. This frog was once known from mid-eastern New South Wales to eastern Victoria, and evidence from wildlife atlas databases and targeted searches indicate that it has declined in large portions of its former range, leaving several populations that are isolated, in some cases restricted in distribution, and of small size. We investigated the relationships among populations using mitochondrial *ND4* nucleotide sequences and single nucleotide polymorphisms (SNPs) from the nuclear genome. We found that northern and southern populations form two highly divergent genetic groups whose distributions abut at the southern margin of the Sydney Basin Bioregion and these genetic groups also show divergence in morphology and male advertisement calls. Here we describe the populations to the south of the Sydney Basin Bioregion as a new species and provide information on its distribution and ecology. In light of the apparent isolation and small size of known populations of the new species and the consequent restriction of the range of *L. littlejohni*, we assessed the conservation status of both species.

Key words: Amphibia, Anura, Pelodryadidae

Introduction

The *Litoria ewingii* species group comprises six species of frogs (*Litoria ewingii* Dumeril & Bibron 1841, *Litoria jervisiensis* Dumeril & Bibron 1841*, Litoria littlejohni* White, Whitford & Mahony 1994*, Litoria paraewingi* Watson, Loftus-Hills & Littlejohn 1971*, Litoria revelata* Ingram, Corben & Hosmer 1982*,* and *Litoria verreauxii* Dumeril 1853), which are found in moist habitats associated with the Great Dividing Range, tablelands, and riverine flood plains of eastern Australia including eastern South Australia, Kangaroo Island and Tasmania. Several members of this group have been investigated extensively as a model system to illustrate the evolution of character displacement (= reinforcement) and the role of the male advertisement call in pre-mating isolation in geographic speciation (Littlejohn 1965, Littlejohn & Loftus-Hills 1968, Loftus-Hills & Littlejohn 1971, Littlejohn 1976, Littlejohn & Watson 1985). It is therefore reasonable to suspect that the taxonomy of this species group would be well characterized. However, several members of the groups have received little systematic attention, and to some degree, this is because they are rarely encountered.

Litoria littlejohni is rare in museum collections and has been encountered rarely in surveys over the past three decades (Gillespie *et al.* 2016, Lemckert 2010). Infection of *L. littlejohni* by the introduced amphibian pathogen *Ba-*

Accepted by J. Rowley: 3 Sept. 2020; published: 30 Sept. 2020

trochochytrium dendrobatidis (chytrid) has been observed (DEEC 2007). While empirical evidence that chytrid is pathogenic in *L. littlejohni* is absent currently, the knowledge that numerous Australian amphibians are susceptible to the disease chytridiomycosis caused by chytrid (Berger *et al*. 1998, Skerratt *et al*. 2007) leads to the possibility that the low detection of populations is due to this threatening process. A wide distribution and an apparent natural geographic disjunction in mid-eastern NSW (Gillespie *et al.* 2016), prompts scrutiny of the systematic status of *L. littlejohni* as these attributes are associated often with the presence of cryptic species (Donnellan & Aplin 1989, Donnellan *et al.* 1993). Identification of cryptic diversity also has important implications for assigning conservation status to individual species. The presence of cryptic species can significantly affect the assessment of the impact of threatening processes, in particular the impact of the loss of genetic diversity and population fragmentation.

In order to assess the species status of populations of *L. littlejohni*, we applied molecular genetic approaches and examined variation in morphology and male advertisement calls. Our analyses support recognition of a northern and a southern species, the latter that we describe as a new species.

Materials and methods

Mitochondrial DNA. We obtained nucleotide sequences of the mitochondrial *ND4* gene from 22 *Litoria littlejohni* and a range of the outgroups *L. ewingii, L. verreauxii, L. jervisiensis, and L. revelata* (Fig. 1, Table 1). DNA was extracted from liver, muscle, skin biopsy or skin swabs with a Gentra Purgene kit (Qiagen). The *ND4* gene was PCR amplified and directly sequenced with the primers: 5'-TGA CTA CCA AAA GCT CAT GTA GAA GC-3' and 5'-GGT YAC GAG YAA TTA GCA GTT CT-3', using protocols detailed in Anstis *et al.* (2016). Sequences were aligned with Muscle v6.814b (Edgar 2004) implemented in Geneious Pro v8.1.4 (Kearse *et al.* 2012) and GenBank accession numbers are listed in Table 1.

Bayes factors were used to assess all possible alternative partitioning strategies for four data subsets—1st, 2nd and 3rd codon positions and the tRNA in PartitionFinder v1.0.0 (Lanfear *et al.* 2017). The Bayes Information Criterion (BIC) were used to assess the best fit partition strategy and nucleotide substitution model for each data subset in the selected partition strategy. Sequences were analysed phylogenetically using Bayesian and maximum likelihood methods. Bayesian analysis was conducted using MrBayes v3.2.7 (Ronquist *et al.* 2012). The analysis was run with model parameters unlinked using default priors for 10 million generations with two independent runs and two chains sampling every 1000 generations. Convergence was assessed as achieved when the average standard deviation of split frequencies was <0.001 and effective sample sizes (ESS) were >200 as determined in TRACER v1.7 (Rambaut *et al.* 2018). The first 25% of sampled trees were discarded as burn-in. Partitioned maximum likelihood (ML) analysis was performed using RAxML v8.0 (Stamatakis 2014) on the CIPRES Science Gateway (Miller *et al.* 2010).

Net average sequence divergence between lineages (*d*A) was calculated in MEGA v7 (Kumar *et al.* 2016) as: *d*A = *d*XY - (*d*X + *d*Y)/2, where, *d*XY is the average distance between groups X and Y, and *d*X and *d*Y are the within-group mean.

Molecular diagnostics. Following the recommendation of Renner (2016), we visually identified diagnostic SNPs within the mitochondrial *ND4* gene in MEGA v7 (Kumar *et al.* 2016). We selected the apomorphic diagnostic SNPs for each species, using the outgroups to assess character state polarity.

SNP genotyping methods. Samples were submitted for DNA extraction and DArTseq™ 1.0 genotyping at Diversity Arrays Technology PL, Canberra, ACT, Australia. DArTseq™ represents a combination of DArT genome complexity reduction methods and next generation sequencing platforms (Kilian *et al*. 2012, Courtois *et al*. 2013, Raman *et al*. 2014, Cruz *et al*. 2013). DNA samples were processed in digestion/ligation reactions using the restriction enzyme combination of *Pst*I/*Sph*I as described by Kilian *et al*. (2012) except that the single *Pst*I-compatible adaptor was replaced with two different adaptors corresponding to the *Pst*I and *Sph*I restriction enzyme overhangs. The *Pst*I compatible adapter was designed to include the Illumina flow cell attachment sequence, sequencing primer and a 'staggered', varying length barcode region, similar to the sequence previously reported (Elshire *et al*. 2011). The *Sph*I-compatible adapter comprised the Illumina flow cell attachment region and *Sph*I overhang sequence.

Ligated fragments with both a *Pst*I and *Sph*I adapter were amplified by PCR using an initial denaturation step of 94°C for 1min, followed by 30 cycles with the following temperature profile: denaturation at 94°C for 20 s, annealing at 58°C for 30 s and extension at 72°C for 45 s, with an additional final extension at 72°C for 7min. Equimolar

amounts of amplification products from each sample were combined before single end sequencing for 77 cycles on an Illumina Hiseq2500.

The raw sequence data were converted to .fastq files using the Illumina HiSeq2500 software. Sequences generated from each lane were processed using proprietary DArT analytical pipelines. In the primary pipeline the fastq files were first processed to filter away poor-quality sequences, with application of more stringent selection criteria to the barcode region compared to the rest of the sequence. In that way the assignments of the sequences to specific samples carried in the barcode allocation step were very reliable. Sequences from each sample were collected, separated by individuals, stripped of barcodes, cleaned and filtered to include only those with a Phred score ≥ 25. Subsequently, sequences were aligned and matched to catalogued sequences in both NCBI GenBank and DArTdb custom databases to check for viral and bacterial contamination, with any matches removed from further processing. Identical sequences are collapsed into 'fastqcall' files.

The fastqcall' files are used in the secondary pipeline implementing proprietary SNP calling algorithms in DArTSoft14™ (Diversity Arrays Technology). Low quality base calls in singleton tags in the fastqcall files were assigned correct base calls using collapsed tags with multiple members as a template. For SNP calling all tags from all libraries included in the DArTsoft14 analysis were clustered using DArT PL's C++ algorithm at the threshold Hamming distance of 3bp, followed by parsing of the clusters into separate SNP loci using a range of technical parameters, especially the balance of read counts for the allelic pairs. All monomorphic sequence clusters were removed and SNPs were called only if they were present in both homozygous and heterozygous forms. One third of samples were processed twice from DNA, using independent adaptors, to allelic calls as technical replicates. Scoring consistency (repeatability) was used as the main selection criterion for high quality/ low error rate markers. The average read depth across loci was 7.9 reads per individual per locus for reference alleles and 6 for SNP alleles.

The data were converted to a matrix of SNP loci by individuals, with the contents stored as integers 0, homozygote, reference state; 1, heterozygote; and 2, homozygote for the alternate state. DNA sequences and statistics (i.e., call rate, polymorphic information content, heterozygosity, read depth, and reproducibility for all loci and individuals) are accessible from Diversity Array Technology Pty. Ltd., Canberra, Australia (Report-DLit19-4642).

Additional SNP filtering. The SNP data and associated metadata were read into a genlight object (Jombart 2008) to facilitate processing with package dartR (Gruber *et al*. 2018). Further filtering was undertaken on the basis of call rate (98% unless otherwise specified). We filtered out secondary SNPs where they occurred in a single sequenced tag, retaining only one SNP from each tag at random. Any monomorphic loci arising as a result of the removal of individuals were also deleted. Given the low within-population sample sizes ($n \leq 15$), we did not filter loci for departures from Hardy-Weinberg equilibrium (HWE) or linkage disequilibrium. For the SVDQuartets analysis to retain *L. revelata* outgroups, we filtered loci at 0.9 and individuals at 0.50, which produced 8534 SNPs for 85 individuals.

Analysis of the SNP data. We used two approaches to identify genetic clusters from the SNP data. Initially, genetic similarity among individuals was visualized using the principal coordinates analysis (PCoA) ordination method as implemented in the gl.pcoa and gl.pcoa.plot functions of dartR. We used a scree plot of eigenvalues to assess the number of informative PCs to examine, based on the average percentage variation the in the original variables explained by the PCs, using the gl.pcoa.scree function in dartR.

Secondly, we used the Bayesian clustering approach implemented in STRUCTURE (Pritchard *et al*. 2000) to identify clusters of individuals corresponding to the uppermost hierarchical level and has been shown to perform well with codominant markers such as SNPs. We used the uncorrelated allele frequency and the admixture ancestry models with prior locality information to assess values of K from 1 to 5. We performed 3 independent runs with 20,000 burnin and 50,000 MCMC iterations for each value of K. The preferred value of K was determined using the change in the second order of likelihood, ΔK (Evanno *et al.* 2005) in Structure Harvester webserver (Earl 2012). We then ran 10 independent runs with the preferred K for 20,000 burnin and 100,000 MCMC iterations and summarised the individual ancestries across all 10 runs in CLUMPAK (Kopelman *et al*. 2015).

We assessed divergence between clusters identified in the PCoA and STRUCTURE by the determining the proportion of loci showing fixed allelic differences between the clusters. Fixed difference at a locus occurs when two populations share no alleles. When many loci are examined and sample sizes are finite, fixed differences will occur through sampling error. We used simulations implemented in dartR (Georges *et al*. 2018) to estimate the expected false positive rate in pairwise comparisons. We used a tloc=0.05 meaning that SNP allele frequencies of 95,5 and 5,95 percent were regarded as fixed when comparing two populations at a locus.

TABLE 1. Specimens examined genetically. Localities are in New South Wales unless indicated otherwise. M—*ND2* mtDNA, R—SNPs, H-NRC—Hawkesbury-Nepean River Catchment, SCA—State Conservation Area.

Molec	ABTC	Species	Location number and location	Lat	Long	GenBank Acc	RegNum
R	7138	*littlejohni*	1 Watagan National Park	-33.026	151.378		SAMA R19793
R	7139	*littlejohni*	1 Watagan National Park	-33.026	151.378		SAMA R19794
R	7140	*littlejohni*	1 Watagan National Park	-33.026	151.378		SAMA R19795
M	97411	*littlejohni*	1 Olney SF, Watagan Mountains	-33.067	151.339	MT497831	No voucher
M	97414	*littlejohni*	1 Olney SF, Watagan Mountains	-33.067	151.339	MT497832	No voucher
M	80813	*littlejohni*	1 Sawmill Road, Watagan Mountains	-33.083	151.357	MT497839	No voucher
M	97410	*littlejohni*	1 Olney SF, Watagan Mountains	-33.098	151.35	MT497833	No voucher
M	97412	*littlejohni*	1 Olney SF, Watagan Mountains	-33.098	151.35	MT497837	No voucher
M	97413	*littlejohni*	1 Olney SF, Watagan Mountains	-33.098	151.35	MT497834	No voucher
M	97415	*littlejohni*	1 Olney SF, Watagan Mountains	-33.098	151.35	MT497838	No voucher
M	97416	*littlejohni*	1 Olney SF, Watagan Mountains	-33.098	151.35	MT497835	No voucher
M	97417	*littlejohni*	1 Olney SF, Watagan Mountains	-33.098	151.35	MT497836	No voucher
R	150904	*littlejohni*	2 Kings Tableland, Andersons Fire Trail	-33.803	150.426		No voucher
M	150907	*littlejohni*	2 Kings Tableland, Andersons Fire Trail	-33.803	150.426	MT497840	No voucher
M	150908	*littlejohni*	2 Kings Tableland, Andersons Fire Trail	-33.803	150.426	MT497841	No voucher
R	150909	*littlejohni*	2 Kings Tableland, Andersons Fire Trail	-33.803	150.426		No voucher
R	150910	*littlejohni*	2 Kings Tableland, Andersons Fire Trail	-33.803	150.426		No voucher
M	145264	*littlejohni*	3 Kings Tableland, Red Ridge Road North	-33.814	150.38	MT497842	No voucher
R	145265	*littlejohni*	3 Kings Tableland, Red Ridge Road North	-33.814	150.38		No voucher
R	145266	*littlejohni*	3 Kings Tableland, Red Ridge Road North	-33.814	150.38		No voucher
R	145267	*littlejohni*	3 Kings Tableland, Red Ridge Road North	-33.814	150.38		No voucher
R	145268	*littlejohni*	3 Kings Tableland, Red Ridge Road North	-33.814	150.38		No voucher
M	145269	*littlejohni*	3 Kings Tableland, Red Ridge Road North	-33.814	150.38	MT497843	No voucher
R	113929	*littlejohni*	4 Woronora River	-34.188	150.899		No voucher
R	113930	*littlejohni*	4 Woronora River	-34.188	150.899		No voucher
R	113931	*littlejohni*	4 Woronora River	-34.188	150.899		No voucher
R	113932	*littlejohni*	4 Woronora River	-34.188	150.899		No voucher
R	113933	*littlejohni*	4 Woronora River	-34.188	150.899		No voucher

.....continued on the next page

TABLE 1. (Continued)

Molec	ABTC	Species	Location number and location	Lat	Long	GenBank Acc	RegNum
R	113919	*littlejohni*	5 Dharawal National Park	-34.197	150.859		No voucher
R	113920	*littlejohni*	5 Dharawal National Park	-34.197	150.859		No voucher
R	113921	*littlejohni*	5 Dharawal National Park	-34.197	150.859		No voucher
R	113922	*littlejohni*	5 Dharawal National Park	-34.197	150.859		No voucher
R	113941	*littlejohni*	6 Dharawal National Park	-34.221	150.862		No voucher
M	140300	*littlejohni*	6 Dharawal National Park	-34.221	150.885	MT497844	AMS R.183346
R	145098	*littlejohni*	6 Dharawal National Park	-34.221	150.862		No voucher
R	145099	*littlejohni*	6 Dharawal National Park	-34.221	150.862		No voucher
R	145100	*littlejohni*	6 Dharawal National Park	-34.221	150.862		No voucher
R	145101	*littlejohni*	6 Dharawal National Park	-34.221	150.862		No voucher
R	113945	*littlejohni*	7 Dharawal National Park	-34.231	150.851		No voucher
R	113946	*littlejohni*	7 Dharawal National Park	-34.231	150.851		No voucher
R	113947	*littlejohni*	7 Dharawal National Park	-34.231	150.851		No voucher
R	113948	*littlejohni*	7 Dharawal National Park	-34.231	150.851		No voucher
R	113949	*littlejohni*	7 Dharawal National Park	-34.231	150.851		No voucher
R	113918	*littlejohni*	8 Cataract River, H-NRC	-34.274	150.765		No voucher
R	145087	*littlejohni*	8 Cataract River, H-NRC	-34.274	150.765		No voucher
R	145088	*littlejohni*	8 Cataract River, H-NRC	-34.274	150.765		No voucher
R	145089	*littlejohni*	8 Cataract River, H-NRC	-34.274	150.765		No voucher
R	145090	*littlejohni*	8 Cataract River, H-NRC	-34.274	150.765		No voucher
R	113938	*littlejohni*	9 Avon River, H-NRC	-34.327	150.678		No voucher
R	113939	*littlejohni*	9 Avon River, H-NRC	-34.327	150.678		No voucher
R	145096	*littlejohni*	9 Avon River, H-NRC	-34.327	150.678		No voucher
R	145097	*littlejohni*	9 Avon River, H-NRC	-34.327	150.678		No voucher
R	113926	*littlejohni*	10 Donalds Castle Creek, H-NRC	-34.361	150.69		No voucher
R	113927	*littlejohni*	10 Donalds Castle Creek, H-NRC	-34.361	150.69		No voucher
R	113928	*littlejohni*	10 Donalds Castle Creek, H-NRC	-34.361	150.69		No voucher
R	113924	*littlejohni*	11 lower Cordeaux River catchment, H-NRC	-34.362	150.688		No voucher

.....continued on the next page

TABLE 1. (Continued)

Molec	ABTC	Species	Location number and location	Lat	Long	GenBank Acc	RegNum
R	113925	*littlejohni*	11 lower Cordeaux River catchment, H-NRC	-34.362	150.688		No voucher
R	113923	*littlejohni*	12 Lake Cordeaux, H-NRC	-34.366	150.743		No voucher
R	113934	*littlejohni*	13 Cordeaux River, H-NRC	-34.439	150.743		No voucher
R	113935	*littlejohni*	13 Cordeaux River, H-NRC	-34.439	150.743		No voucher
R	113936	*littlejohni*	13 Cordeaux River, H-NRC	-34.439	150.743		No voucher
R	113937	*littlejohni*	13 Cordeaux River, H-NRC	-34.439	150.743		No voucher
R	145095	*littlejohni*	13 Cordeaux River, H-NRC	-34.439	150.743		No voucher
R	113917	*littlejohni*	14 Dudewaugh Creek, Upper Nepean SCA	-34.527	150.643		No voucher
R	113942	*littlejohni*	14 Dudewaugh Creek, Upper Nepean SCA	-34.527	150.643		No voucher
R	113943	*littlejohni*	14 Dudewaugh Creek, Upper Nepean SCA	-34.527	150.643		No voucher
R	113944	*littlejohni*	14 Dudewaugh Creek, Upper Nepean SCA	-34.527	150.643		No voucher
R	145086	*littlejohni*	14 Dudewaugh Creek, Upper Nepean SCA	-34.527	150.643		No voucher
RM	149194	*watsoni*	15 Gerringong Falls, Budderoo National Park	-34.661	150.654	MT497845	AMS R186902
R	113907	*watsoni*	16 Barren Grounds Nature Reserve	-34.674	150.705		No voucher
R	113908	*watsoni*	16 Barren Grounds Nature Reserve	-34.674	150.705		No voucher
R	113909	*watsoni*	16 Barren Grounds Nature Reserve	-34.674	150.705		No voucher
R	113910	*watsoni*	16 Barren Grounds Nature Reserve	-34.674	150.705		No voucher
R	113911	*watsoni*	16 Barren Grounds Nature Reserve	-34.674	150.705		No voucher
R	113912	*watsoni*	16 Barren Grounds Nature Reserve	-34.674	150.705		No voucher
R	113913	*watsoni*	16 Barren Grounds Nature Reserve	-34.674	150.705		No voucher
R	113914	*watsoni*	16 Barren Grounds Nature Reserve	-34.674	150.705		No voucher
R	113915	*watsoni*	16 Barren Grounds Nature Reserve	-34.674	150.705		No voucher
R	113916	*watsoni*	16 Barren Grounds Nature Reserve	-34.674	150.705		No voucher
R	145084	*watsoni*	16 Barren Grounds Nature Reserve	-34.674	150.705		No voucher
R	145085	*watsoni*	16 Barren Grounds Nature Reserve	-34.674	150.705		No voucher
R	113905	*watsoni*	17 Parma Creek, Parma Creek Nature Reserve	-35.02	150.496		No voucher
R	113906	*watsoni*	17 Parma Creek, Parma Creek Nature Reserve	-35.02	150.496		No voucher
R	145077	*watsoni*	17 Parma Creek, Parma Creek Nature Reserve	-35.02	150.496		No voucher

.....continued on the next page

TABLE 1. (Continued)

Molec	ABTC	Species	Location number and location	Lat	Long	GenBank Acc	RegNum
R	145277	*watsoni*	17 Parma Creek, Parma Creek Nature Reserve	-35.02	150.496		No voucher
R	149182	*watsoni*	17 Parma Creek, Parma Creek Nature Reserve	-35.02	150.496		AMS R186898
R	149183	*watsoni*	17 Parma Creek, Parma Creek Nature Reserve	-35.02	150.496		AMS R186899
R	149184	*watsoni*	17 Parma Creek, Parma Creek Nature Reserve	-35.02	150.496		AMS R186900
R	149185	*watsoni*	17 Parma Creek, Parma Creek Nature Reserve	-35.02	150.496		AMS R186901
M	140298	*watsoni*	17 Flat Rock Creek, Parma Creek Nature Reserve	-35.028	150.498	MT497846	AMS R177178
M	140299	*watsoni*	17 Flat Rock Creek, Parma Creek Nature Reserve	-35.028	150.498	MT497847	AMS R177179
RM	17597	*watsoni*	18 5km NE Tianjara Falls	-35.1	150.37	MT497848	SAMA R42607
R	145080	*watsoni*	19 Tianjara Falls, Morton National Park	-35.111	150.331		No voucher
R	145081	*watsoni*	19 Tianjara Falls, Morton National Park	-35.111	150.331		No voucher
R	145082	*watsoni*	19 Tianjara Falls, Morton National Park	-35.111	150.331		No voucher
R	150911	*watsoni*	20 Yadboro SF, corner of Western Distributor and Mares Rd	-35.415	150.128		No voucher
R	150912	*watsoni*	20 Yadboro SF, corner of Western Distributor and Mares Rd	-35.415	150.128		No voucher
R	150913	*watsoni*	20 Yadboro SF, corner of Western Distributor and Mares Rd	-35.415	150.128		No voucher
R	150914	*watsoni*	20 Yadboro SF, corner of Western Distributor and Mares Rd	-35.415	150.128		No voucher
R	150916	*watsoni*	21 Merrica River Road, Nadgee SF	-37.387	149.853		No voucher
M	139706	*watsoni*	22 Yalmy Road, 5.2km SSW Bonang, Vic	-37.226	148.704	MT497849	No voucher
M	139703	*watsoni*	23 Mount Jersey Road, 14km SSW Bonang, Vic	-37.29	148.64	MT497850	No voucher
M	139704	*watsoni*	23 Mount Jersey Road, 14km SSW Bonang, Vic	-37.29	148.64	MT497851	No voucher
M	139705	*watsoni*	23 Mount Jersey Road, 14km SSW Bonang, Vic	-37.29	148.64	MT497852	No voucher
M	12436	*ewingii*	3km E Toora, Vic	-38.67	146.37	MT497817	SAMA R39005
M	37533	*ewingii*	20km WNW Millicent Airport, SA	-37.51	140.15	MT497818	SAMA R49588
M	25451	*jervisiensis*	Darkes Forest	-34.24	150.93	MT497819	
M	25839	*jervisiensis*	Mungo Brush, Myall Lakes National Park	-32.52	152.32	MT497820	
M	12855	*paraewingi*	7km N Merton, Vic	-36.93	145.75	MT497821	SAMA R44074
M	12856	*paraewingi*	Polly McQuinn Weir, 6km W Strathbogie, Vic	-36.88	145.68	MT497822	SAMA R44066
M	40923	*paraewingi*	Lima Turn-off on Midland Hwy, Vic	-36.6	145.95	MT497823	SAMA R34675
RM	102390	*revelata*	Zillie Falls Road, Qld	-17.47	145.65	MT497815	

.....continued on the next page

TABLE 1. (Continued)

Molec	ABTC	Species	Location number and location	Lat	Long	GenBank Acc	RegNum
RM	102391	*revelata*	Zillie Falls Road, Qld	-17.47	145.65	MT497816	
M	17600	*revelata*	Ourimbah State Forest	-33.33	151.32	MT497824	SAMA R42610
M	25931	*revelata*	Lamington National Park, Qld	-28.21	153.12	MT497825	
R	80817	*revelata*	Dalrymple Road, Eungella, Qld	-21.03	148.59		
R	80818	*revelata*	Dalrymple Road, Eungella, Qld	-21.03	148.59		
R	81985	*revelata*	Mount William Creek Eungella National Park, Qld	-21.03	148.6		
R	90388	*revelata*	Mt William Creek, Eungella National Park, Qld	-21.03	148.6		
R	102392	*revelata*	Zillie Falls Road, Qld	-17.47	145.65		
M	28896	*rothi*	Black Point, NT	-11.15	132.15	MT497813	MAGNT R29049
M	28898	*rothi*	Black Point, NT	-11.15	132.15	MT497814	MAGNT R26982
M	29374	*rothi*	Cape Crawford, NT	-16.683	135.717	MT497812	MAGNT R20539
M	16902	*rubella*	Borroloola, NT	-16.07	136.3	MT497810	SAMA R38511
M	28899	*rubella*	Black Point, NT	-11.15	132.15	MT497811	MAGNT R29055
M	26497	*verreauxii*	Mt Royal National Park	-34.249	151.275	MT497826	-
M	24897	*verreauxii*	Olney SF, Watagan Mountains	-33.067	151.339	MT497827	-
M	1161	*verreauxii*	Ulladulla	-35.35	150.483	MT497829	AMS R130059
M	12630	*verreauxii*	Nepean Hall, Camden Campus of Sydney Univ.	-34.05	150.67	MT497828	SAMA R40839
M	86384	*verreauxii*	Bairnsdale, Vic	-37.83	147.62	MT497830	ANWC A02315

We inferred phylogenetic relationships among the samples using the concatenated SNP data set with two phylogenetic tree building methods suited to SNP data, SVDquartets and maximum likelihood. SVDquartets (Chifman & Kubatko 2014) accounts for differences in the genealogical histories of individual loci and for sequence variability due to both mutational and coalescent variance. In addition, the method is rapid and results are straightforward to interpret, in contrast to other SNP-based approaches that use MCMC approaches, e.g., SNAPP (Bryant *et al*. 2012), which can be slow for large data sets and difficult to assess convergence. A large number of quartets must be sampled to estimate phylogenetic relationships. We used *L. revelata* as the outgroup. Three independent runs of SVDquartets with sampling of 100,000 randomly selected quartets were conducted in the program PAUP* version 4.0a build 165 (Swofford 2003) to assess topological convergence, each of which included 500 bootstrap replicates.

For the maximum likelihood approach, we used IQ-tree (Nguyen *et al.* 2014), with the Lewis-type ascertainment bias correction, on the IQ-TREE webserver (Trifinopoulos *et al*. 2016). The ascertainment bias correction considers that no invariant sites are included in the data and helps reduce overestimation of tree lengths (Leaché *et al.* 2015). Heterozygous SNPs were coded as the appropriate IUPAC ambiguity codes. We estimated the best substitution model with ModelFinder (Kalyaanamoorthy *et al*. 2017) following the BIC criterion. We assessed branch support with 1000 ultrafast bootstrap pseudo-replicates (Hoang *et al.* 2017).

Morphological Analyses. Morphometric measurements of preserved adult specimens were taken with callipers to the nearest mm following Watters *et al.* (2016). Dimensions measured (in mm) were: snout-vent length (SVL), head width (HW), head length (HL), eye length (EYE), eye to naris distance (EN), internarial span (IN), greatest length of tympanum (TYM), tibia length (TBL), tarsus length, i.e. from ankle to heel (TAL). Males and females were analysed separately. Sex was determined by visual inspection of gonads or the presence of nuptial pads in males.

To compare differences in shape between taxa, we used a multivariate method, a linear discriminant function analysis (DFA). Potentially confounding variation associated with differing body sizes and allometric growth was minimised by scaling measurements to a standard snout-vent length (SVL; the mean value for each sex) using equation 13 of Lleonart *et al.* (2000; p. 88): $y_i^* = y_i(x_o/x_i)^b$, where y_i^* and y_i are, respectively, scaled and measured values of a variable for specimen i, x_o is the standard body size (SVL in this instance) to which measurements are scaled, x_i is the observed body size of specimen i and b is a constant. Values of b were estimated independently for females and males as the within-sex regression coefficient calculated for logarithmically transformed values of x_i and y_i (see Thorpe 1976, Lleonart *et al.* 2000). The analysis notes and embedded R script are available from the authors. For the DFA, we established prior group membership for specimens by choosing those that had either been genotyped or whose collection location fell to the north of Dudewaugh Creek, in the Upper Nepean State Conservation Area for the northern taxon or fell to the south of Gerringong Falls for the southern taxon, i.e. well away from the possible region of contact between the taxa (Fig. 1). We conducted the DFA on the log-transformed metric data using SVL and the scaled versions of the other metric variables as described above using the 'lda' function in RStudio, version 0.98.1028.

Advertisement Call Analysis. Advertisement calls were recorded with a Marantz PMD 660 Recorder (44 kHz sampling rate and 24-bit encoding) with a Røde NTG-2 condenser shotgun microphone and from SM4+ Songmeters set at 32 kHz sampling rate (Wildlife Acoustics). All field recordings were from males in active choruses. Commercial tape recordings (Littlejohn 1987, Grigg & Barker 1973, Stewart 2000), were digitised using a direct line to the Marantz PMD 660 recorder, and the results of previous call analysis were included in comparisons (Martin & Littlejohn 1966, White *et al*. 1980, White *et al*. 1994). All calls were analysed with Raven Pro 1.3© software http://www.birds.cornell.edu/ raven). Audiospectrograms for analysis were calculated with fast-Fourier transform (FFT) of 512 points, 50% overlap and 172 Hz grid-spacing, using Hanning windows, for figures we used 512 points. In describing the advertisement calls, we use the definitions of Köhler *et al*. (2017), and adopt the call centered scheme. For up to five calls per individual male, we measured the call duration (s), intercall interval (s), call repetition rate (calls/s), number of notes per call, note repetition rate (notes/s), the number of pulses in a note and pulse repetition rate (pulse/s), and dominant frequency (Hz). A call recordings has been deposited at the Australian Museum as multimedia record 1682760, attached to the database record for holotype AMS R186898.

The calls of the two taxa are qualitatively very similar, and therefore in comparisons we focus on differences in structural and spectral traits. In a review of calling traits useful for taxonomic purposes Köhler *et al.* (2017) report that the number of notes per call and pulses per note are rather invariable traits, not dependent on temperature or motivation, and thus potentially valuable for taxonomic purposes. Accordingly, we place attention on the differences in the number of notes and pulses. Dominant frequency is also not dependent of temperature or motivation

but is affected by body size. We do not have information on the sizes of all of the males included in the call analysis and therefore we cannot make strong conclusions about differences in dominant frequency. Köhler *et al.* (2017) also observe that among temporal variables, the duration of basic uninterrupted call units (in this case note duration and pulse repetition rate) shows comparatively limited intraspecific variation, and in most cases are not influenced by variation in body size, but are influenced by temperature.

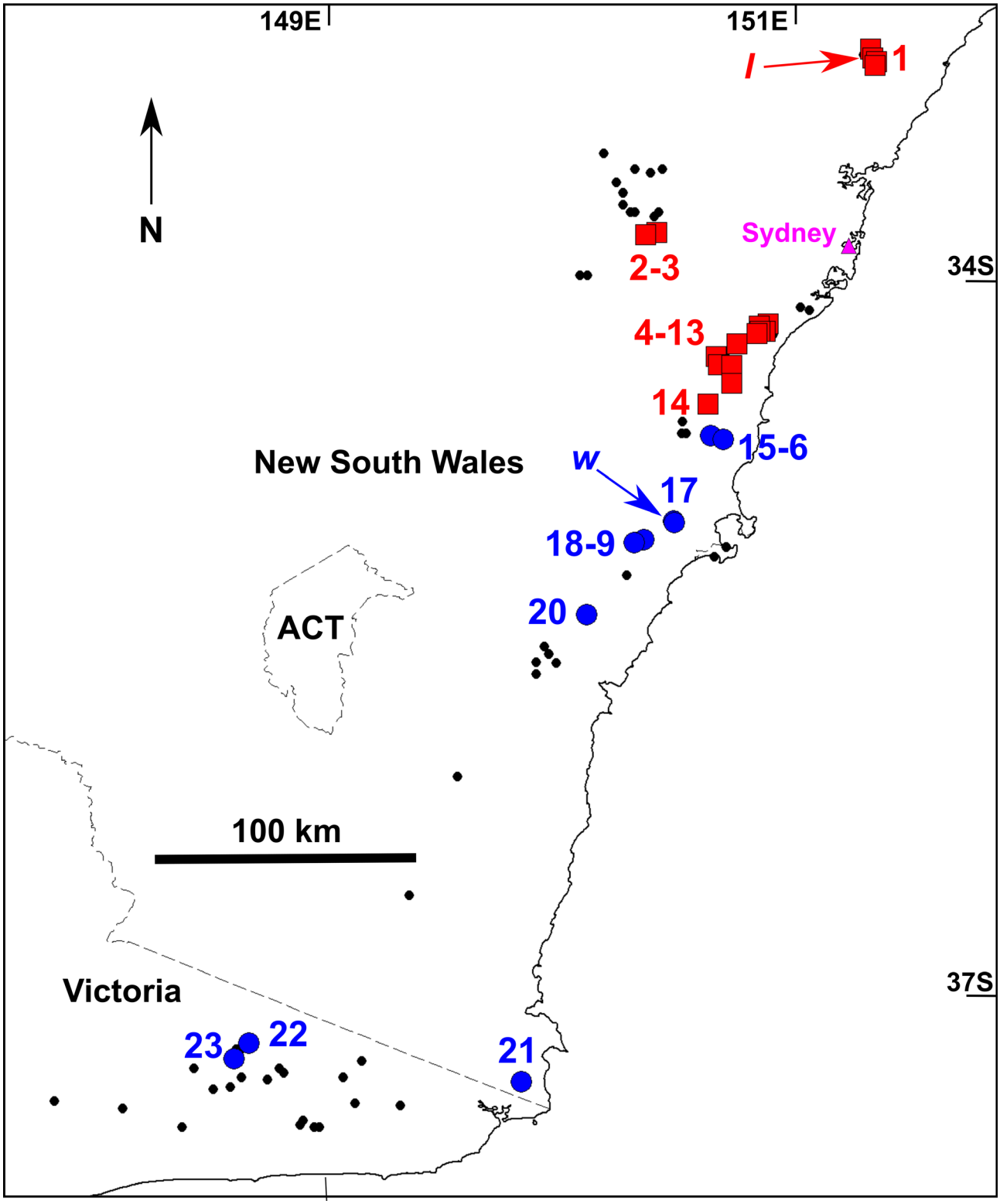

FIGURE 1. Map showing the distribution of genotyped samples of *Litoria littlejohni* [red squares] and *Litoria watsoni* **sp. nov.** [blue circles] and museum voucher records from the Atlas of Living Australia, accessed July 2018 [small black circles]. Symbols arrowed with letters indicate type locations. See Table 1 for key to location numbers.

To address the effect of temperature on call attributes we conducted statistical tests (ANCOVA) with temperature as a covariate. Call attributes were first standardised since the range of temperatures for which calls were recorded for the two species were not the same. There were no significant differences between the taxa for these traits with the exception of pulse repetition rate. For pulse repetition rate we compared slopes of regression lines and then conducted analyses of covariance (ANCOVA) since the slopes were homogenous. For all other variables, we compared samples by ANOVA with post-hoc comparisons amongst means using Scheffe's test.

Conservation assessment. To determine if there have been changes in population distribution and abundance over time we based our analyses on the Area of Occupancy (AOO) and Extent of Occupancy (EOO) (IUCN 2012). To calculate AOO and EOO, we first mapped all records of *L. littlejohni* in the Atlas of Living Australia (ALA—accessed November 2019), and following a process of expert examination, two spurious records were removed from the total of 1973 occurrence records. Second, we divided the records into four time periods; prior to the year 1990, and each decade up till 2020. These periods were chosen since it is reasonably established that the introduced amphibian pathogen *Batrachochytrium dendrobatidis* (chytrid) was present in eastern Australia from at least the mid-1980s (Skerratt *et al*. 2007), and if there was to be an impact from this pathogen on population distribution it should be evident when comparing the pre- and post-1990s periods.

We recognise that this is a broad-brush approach. Although there have been targeted surveys, for example by forestry and mining in specific areas that provide presence records, there are no reports of absences in the data source we used, i.e. the ALA. Thus, more precise evidence of declines is not available. The analysis assumes that all areas have been investigated equally, however, it is apparent that the comprehensiveness of spatial sampling is limited.

Area of occupancy (AOO) was calculated using the IUCN (2012) recommendation for a 2 x 2 km grid cell. Although we have no field information that addresses the distance that adults or tadpoles may disperse, we consider the 2 km grid most likely overestimates the smallest area essential at any life stage to the survival of an existing population (IUCN 2012).

Material examined. See Table 1 and Supplementary Table S2 for details of all material examined.

Results

We use a non-conventional approach to naming species in the results section to make the paper easier for the reader to follow. We use the final specific epithets throughout the manuscript rather than use an initial group nomenclature that we would change to the final specific epithets in the taxonomy section. Of course, we do not assume the separate species status of the two species within *L. littlejohni sensu lato* but rather use the results section to test this hypothesis before dealing with the final taxonomy.

Molecular Genetic Analyses. The mtDNA alignment comprised 786 bp. In the phylogenetic analyses of these data, two main clades are apparent—*L. littlejohni* and *L. watsoni* **sp. nov.** with strong support for each (Fig. 2), and with 5% net average sequence divergence between them (Table 2). In the 786 bp alignment, *L. watsoni* **sp. nov.** is diagnosed by 8 apomorphic diagnostic nucleotide sites and *L. littlejohni* by 12 sites (Table 3).

TABLE 2. Net average sequence divergence between lineages in the *Litoria ewingii* group (*d*A).

	e	j	p	v	r	l	w
ewingii (e)	-						
jervisiensis (j)	0.1	-					
paraewingi (p)	0.09	0.11	-				
verreauxii (v)	0.07	0.1	0.05	-			
revelata (r)	0.1	0.12	0.08	0.07	-		
littlejohni (l)	0.1	0.12	0.07	0.07	0.09	-	
watsoni (w)	0.09	0.12	0.06	0.06	0.09	**0.05**	-

A total of 75,902 polymorphic SNP loci were scored for 91 individuals of *Litoria*. After filtering on call rate (0.9), the "full data set", which included the outgroups, comprised 8,533 polymorphic SNP loci sampled from 74 *L.*

littlejohni sensu lato from 21 locations, and seven individuals of the outgroup *L. revelata* (Table 1). The "full data set" was used for the SVD Quartets analysis. The "ingroup only dataset" comprised 77 individuals of *L. littlejohni sensu lato* from 21 locations with 40,782 polymorphic SNP loci with 70% missing data after filtering on call rate (0.9).

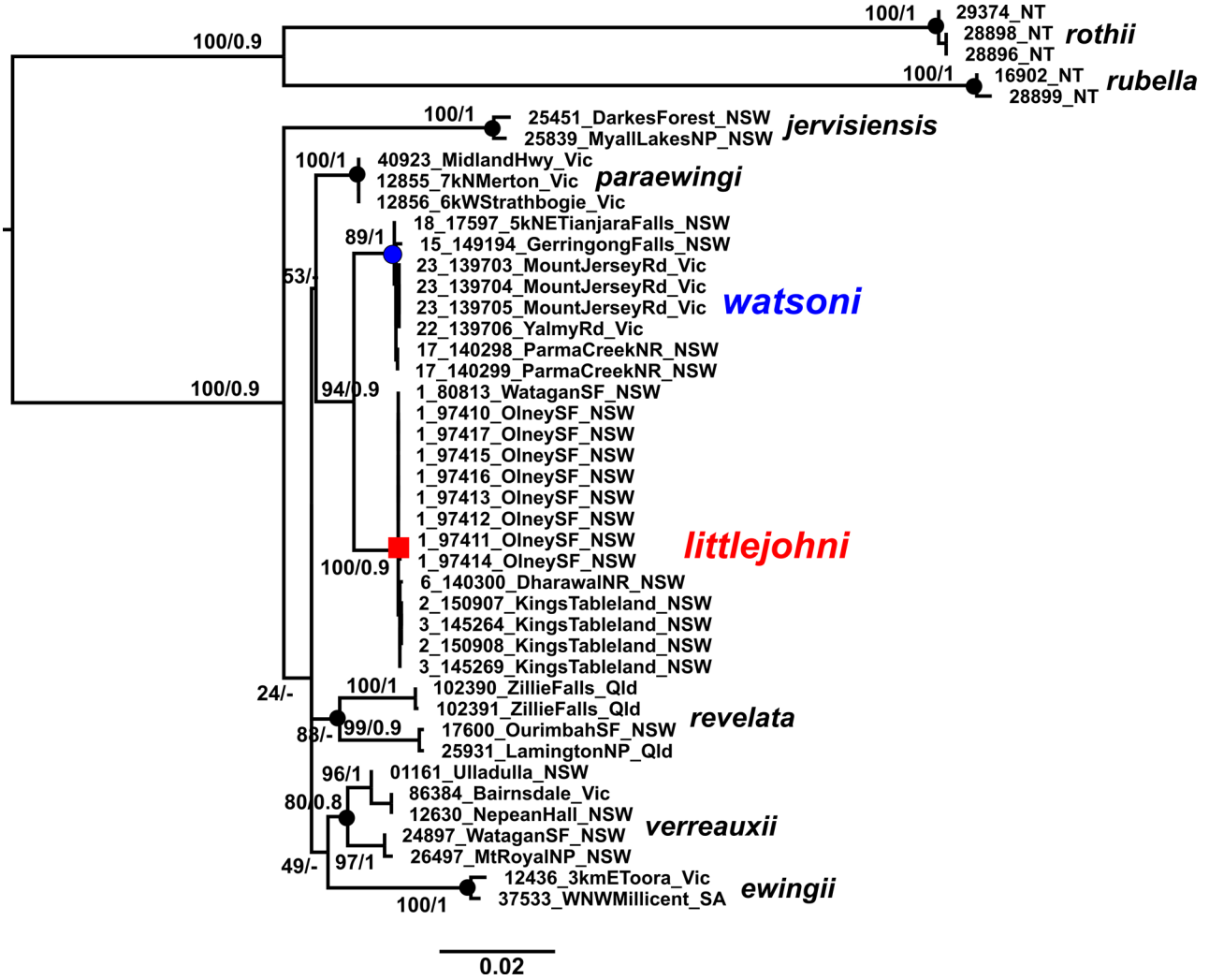

FIGURE 2. Mitochondrial *ND4* ML inference tree for *Litoria littlejohni* and *Litoria watsoni* **sp. nov.** with ML bootstrap proportions (left) and Bayesian posterior probabilities (right) at nodes. The tree was rooted with members of the *Litoria rubella* and *Litoria peronii* species groups. For collection locations and ABTC numbers see Table 1.

TABLE 3. Diagnostic nucleotide sites in mitochondrial *ND4* sequence for species in the *Litoria ewingii* group. Apomorphic states are indicated in bold.

			1	1	1	2	3	3	4	4	4	4	4	5	5	5	5	5	5	7	7
	8	9	5	8	9	5	6	8	1	3	3	8	9	0	1	2	5	6	6	2	6
Taxon	5	7	4	5	6	9	4	8	8	3	7	5	6	3	4	4	9	2	5	1	4
L. watsoni	A	**C**	T	T	T	**T**	T	A	G	**T**	C	**G**	A	C	**C**	**T**	**G**	C	A	**G**	A
L. littlejohni	**T**	T	**C**	**C**	**C**	C	**C**	**G**	**A**	C	**T**	A	**G**	**T**	T	C	A	**T**	**G**	A	**G**
L. paraewingi	A	C	T	T	T	C	T	A	A/G	C	C	A	A	C	T	C	A	C	A	A	A
L. verreauxii	A	T	T	T	T	C	T	A	G	C	C	A	A	C	T	C	A	C	A	A	A
L. jervisiensis	G	T	T	T	T	C	C	A	G	C	C	A	A	C	T	C	A	C	A	A	A
L. revelata	A	T	T	C/T	T	C	T	A	G	C	C	A	A	C/T	C/T	C	C	C/T	A	A	A
L. ewingii	A	T	T	T	T	C	T	A	G	T	T	A	A	C	T	T	A	C	A	A	A

In the initial clustering analysis via PCoA, the proportion of explained variance by the first three PC axes was: 1st axis—14.2%; 2nd axis—5.8%, and 3rd axis—4.84%. Three genetic clusters are apparent in the PCoA: *L. littlejohni* (locations 1–14), northern *L. watsoni* **sp. nov.** (locations 15–16) and southern *L. watsoni* **sp. nov.** (locations 17–19) (Fig. 3A). The STRUCTURE based clustering analysis found two clusters equivalent to *L. littlejohni* (locations 1–14), and *L. watsoni* **sp. nov.** (locations 15–19). (Fig. 3B). The percentage of loci having fixed differences between the three genetic clusters seen in the PCoA analysis ranged from 0.8 to 1.2%, with all values significant after simulation (Table 4).

In the phylogenetic analysis of the "full dataset" based on SVD Quartets, two major well-supported groups, *L. littlejohni* and *L. watsoni* **sp. nov.**, are present in the tree (Fig. 4). The tree also demonstrates a well-supported split within *L. watsoni* **sp. nov.** into the northern and southern groups that was observed also in the PCoA analysis. The maximum likelihood analysis also recovered the two major groups and the northern and southern groups with *L. watsoni* **sp. nov.** with strong support (Supplementary Fig. S1).

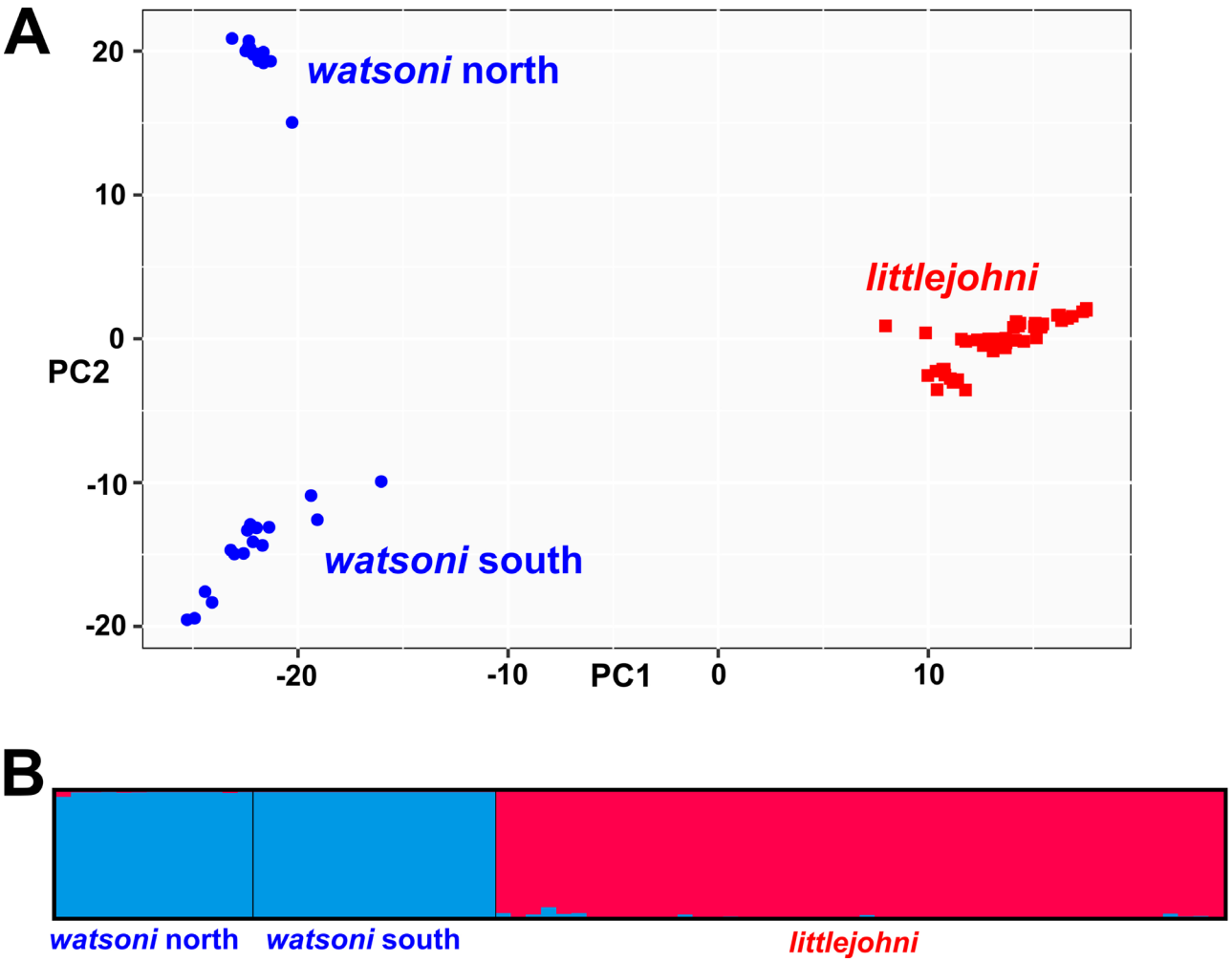

FIGURE 3. Analyses of SNP data for *Litoria littlejohni* and *Litoria watsoni* **sp. nov. A**) PCoA plot based on 10,901 SNPs and **B**) STRUCTURE barplot.

TABLE 4. Fixed difference analysis for *Litoria littlejohni* and *Litoria watsoni* **sp. nov.** (northern and southern generic groups). Upper matrix: number of loci showing a fixed difference and (% of loci showing a fixed difference); lower matrix: expected number of loci showing a fixed difference and (the number of loci compared). All comparisons were significant after simulation.

	L. littlejohni	*L. watsoni*-nth	*L. watsoni*-sth
L. littlejohni	-	453 (1.1%)	496 (1.2%)
L. watsoni-nth	111 (40,132)	-	339 (0.8%)
L watsoni-sth	113 (40,323)	112 (39,829)	-

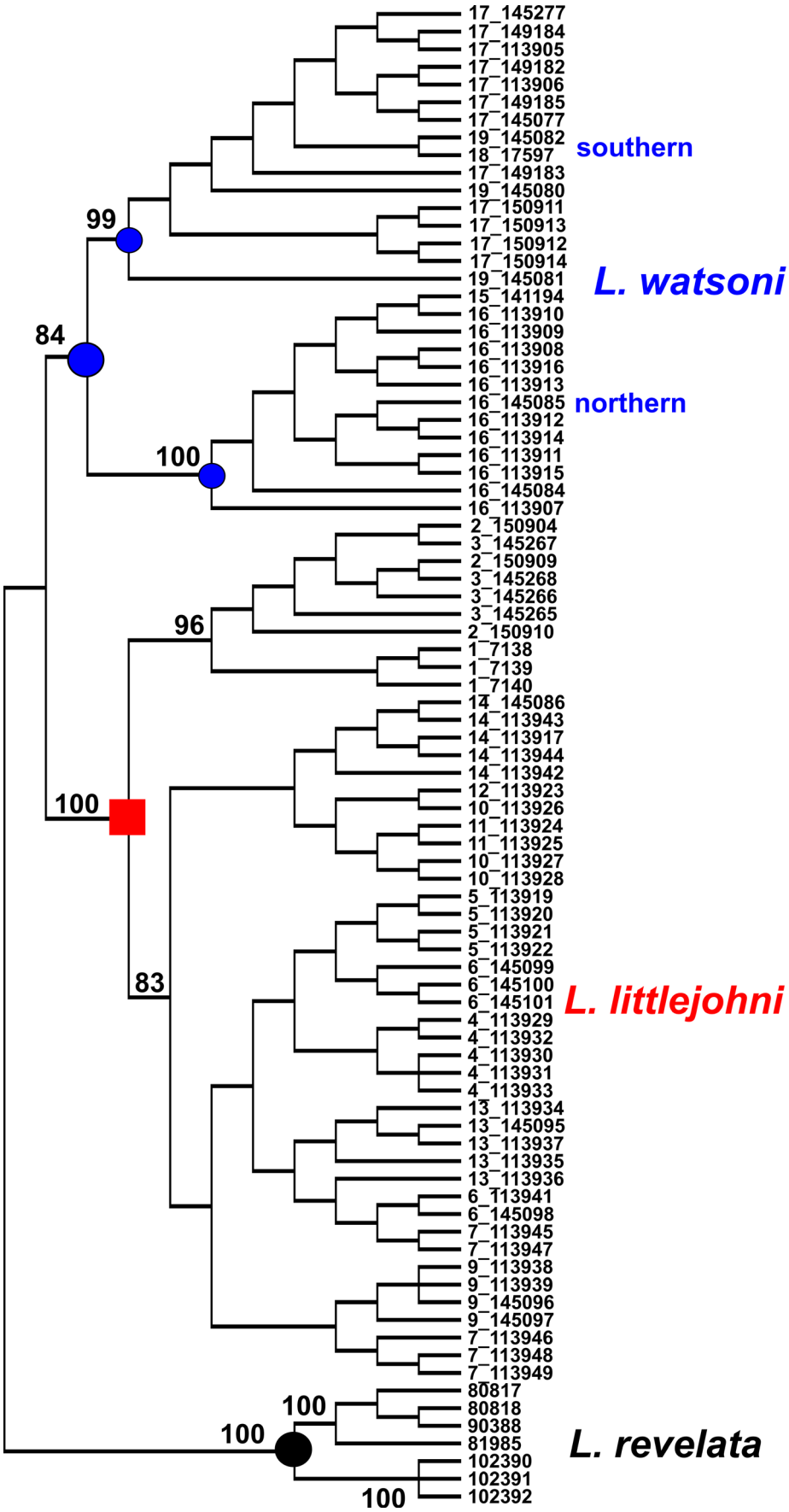

FIGURE 4. SVD Quartets phylogeny for *Litoria* based on SNP genotypes with ambiguity codes substituted for heterozygous sites.

Morphological Analyses. Raw morphometric measurements of adults are summarised in Table 5. The male and female DFAs each returned a single discriminant function (LD). Separation of *L. littlejohni* and *L. watsoni* **sp. nov.** was better for female frogs (Fig. 5). For females (n = 14) overall predictive accuracy was 100% and with jack-knifed validation the classification success was 50%, while for males (n = 69) the overall predictive accuracy was 80% (six *L littlejohni* and eight *L watsoni* **sp. nov.** individuals misclassified) and with jack-knifed validation the classification success was 73%. For males, the traits with the highest coefficients were: EN, TAL and TYM. For females, the traits with the highest coefficients were: HL and SVL.

TABLE 5. Summary of metric variation (mean ±SD and range) in *Litoria littlejohni and L. watsoni* **sp. nov.**

	L. littlejohni		*L. watsoni*	
	female	male	female	male
N	7	25	7	45
SVL	59.1±1.7	48.7±3.5	58.1±5.5	49.9±3.1
	56.9–61.0	43.1–55.1	50.2–63.6	42.2–58.7
HW	20.1±1	16.6±1.2	19.9±1.9	17.2±1.2
	18.9–21.7	14–18.6	16.9–22.5	14.2–20.2
HL	15.2±0.9	13.1±0.9	15.8±1.2	13.8±1.4
	14.1–16.3	11.3–15.5	13.8–17.2	9.4–16.4
EYE	6.1±0.6	5.2±0.5	5.8±0.7	5.4±0.5
	4.9–6.8	4.2–5.9	4.7–6.8	4.2–6.3
EN	4.6±0.4	3.8±0.41	4.9±0.5	4.3±0.4
	4.1–5.1	3.1–4.9	4.3–5.5	3.6–5.6
IN	4.9±0.3	4.2±0.4	4.8±0.7	4.4±0.4
	4.4–5.5	3.3–4.9	3.9–5.9	3.5–5.3
TYM	3.3±0.3	2.9±0.4	3.4±0.3	3.1±0.4
	2.9–3.7	2.0–3.4	2.9–3.8	2.4–4
TBL	31.1±0.8	25.8±1.8	30.8±3.0	26.4±1.3
	29.8–32.1	22.3–29.1	25.6–33.8	23.2–30.3
TAL	19.8±1.1	16.2±1.2	19.3±2.5	16.3±0.9
	17.9–21.1	13.9–18.3	16–22.5	14.5–18.6
HL/HW	0.8±0.03	0.8±0.1	0.8±0.1	0.8±0.1
	0.7–0.8	0.7–0.9	0.73–0.9	0.7–0.9
TBL/SVL	0.5±0.02	0.5±0.02	0.5±0.02	0.5±0.03
	0.5–0.6	0.5–0.6	0.5–0.6	0.4–0.6
TYM/EYE	0.6±0.1	0.6±0.1	0.59±0.1	0.58±0.1
	0.5–0.6	0.4–0.7	0.5–0.7	0.4–0.7

Advertisement Call Analysis. The male advertisement calls of four populations and 11 individuals of *L. littlejohni* and four populations and 11 individuals of *L. watsoni* **sp. nov**. were compared (Supplementary Table S1, Table 6). The calls of the two species are similar in overall temporal and spectral characteristics (Table 6; Fig. 6). While temperature affected several temporal attributes a significant difference in slope in the two regression lines describing the relationship between temperature and the call attribute for each taxon was found only for pulse repetition rate. Regression analysis of pulse repetition rate against taxon, with temperature as a covariate, showed a significant difference (F = 91.5007, P = 0.0001*), *L. watsoni* **sp. nov.** (mean 37.9 pulse/s) and *L. littlejohni* (mean 42.9 pulse/s) (Table 4; Fig. 7). We compared the other attributes using ANOVA, and dominant frequency, number of notes and pulse number differed significantly; *L. watsoni* **sp. nov.** (means; 1740.3 Hz dominant frequency, 22.8 pulses per note, 6.5 notes per call, 37.9 pulse/s) and *L. littlejohni* (means; 1830.4 Hz, 27.8 pulse per note, 8.8 notes, and 42.9 pulse/s) respectively (Table 4).

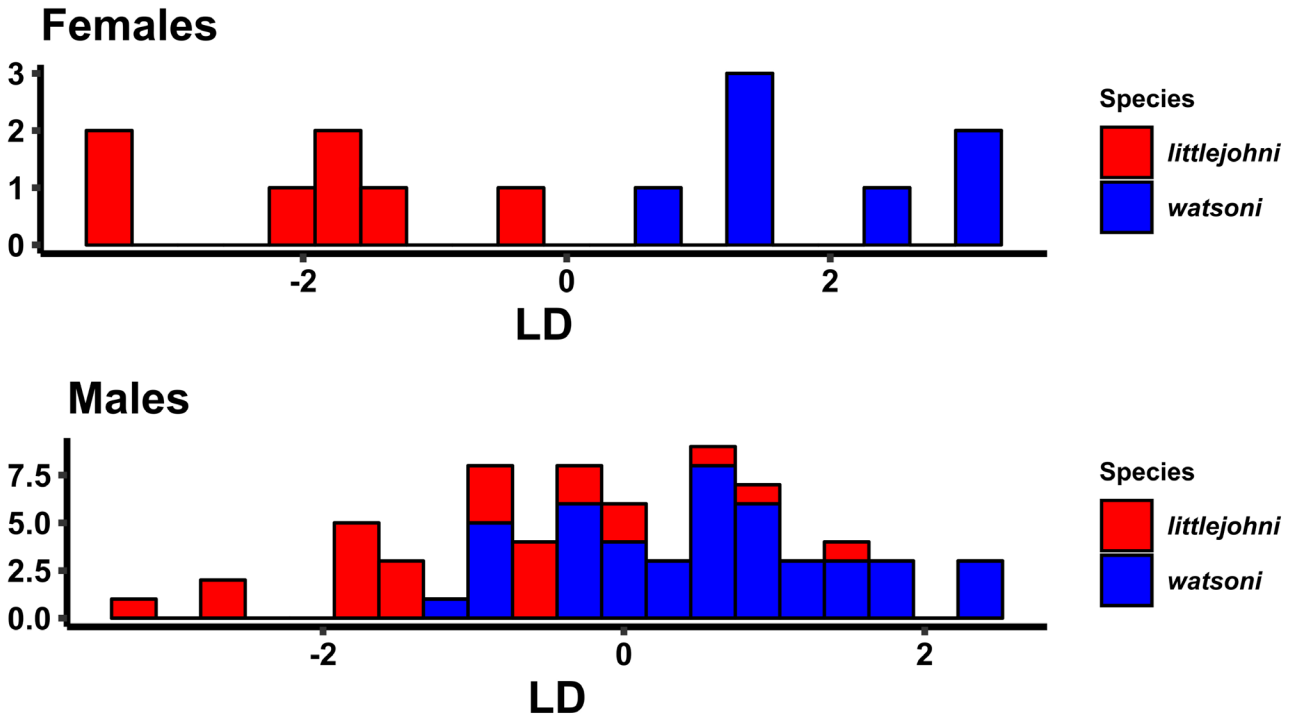

FIGURE 5. Histograms of the discriminant function scores (LD) for morphological analysis of adult female and male frogs.

TABLE 6. Advertisement call parameters, mean±SD, ranges and statistical comparisons for *L. littlejohni* and *L. watsoni* **sp. nov.** Data from four populations and eleven individuals were analysed from each of the two species. Statistical comparisons of pulse repetition rate (ANCOVA) with temperature as a covariate, and ANOVA for the remaining call parameters are included.

	L. littlejohni	*L. watsoni* **sp. nov.**	
	Mean±SD, range	Mean±SD, range	F ratio, P > F
Duration of call (s)	5.8±1.8	5.6±1.8	0.1167, 0.7344
	4.1–9.7	3.4–12.3	
Number of notes	8.8±4.1	6.5±2.0	5.8097, 0.0208*
	5–16	3–14	
Duration of single note (s)	0.63±0.14	0.58±0.22	2.6279, 0.1072
	0.4–1.0	0.2—0.7	
Number of pulses in the 2nd last note	27.8±5.9	22.8±9.8	13.5218, 0.0003*
	16–40	7–33	
Pulse repetition rate (pulses^{-1})	43.25±14.17	37.56±12.2	91.5007, 0.0001*
	34.7–66.7	21.9–60.5	
Dominant Frequency (Hz)	1830±95.6	1740±146.9	7.3741, 0.0087*
	1733–2500	1505–2018	

Systematic Implications. In our genotyped samples, the distributions of *L. littlejohni* and *L watsoni* **sp. nov.** are closely parapatric with the two nearest locations from 400 m a.s.l. at Dudewaugh Creek in the upper catchment of the Nepean River (location 14) for *L. littlejohni* and from 540 m a.s.l. at Gerringong Creek on the Budderoo Plateau (location 15) for *L. watsoni* **sp. nov.**, separated by 10.4 km. These specimens were collected at breeding sites however we have evidence that the frogs disperse widely from these sites based on the observation of specimens under exfoliated rocks on ridge tops (M. Schulz pers com). Both locations are on a continuous raised plateau. There is a relatively small band (<1 km at narrowest point) of continuous forest just below the plateau escarpment between these sites, while much of the land on the plateau has been cleared for agriculture. We consider that the distance

between these two locations is within the lifetime dispersal distance of an adult frog and that their distributions are parapatric, providing the opportunity for gene flow. The presence of a significant proportion of loci showing fixed difference between the groups that is not explained by sampling error is unequivocal evidence for the lack of gene flow between the groups and therefore primary evidence that each comprises separate species (Georges *et al.* 2018).

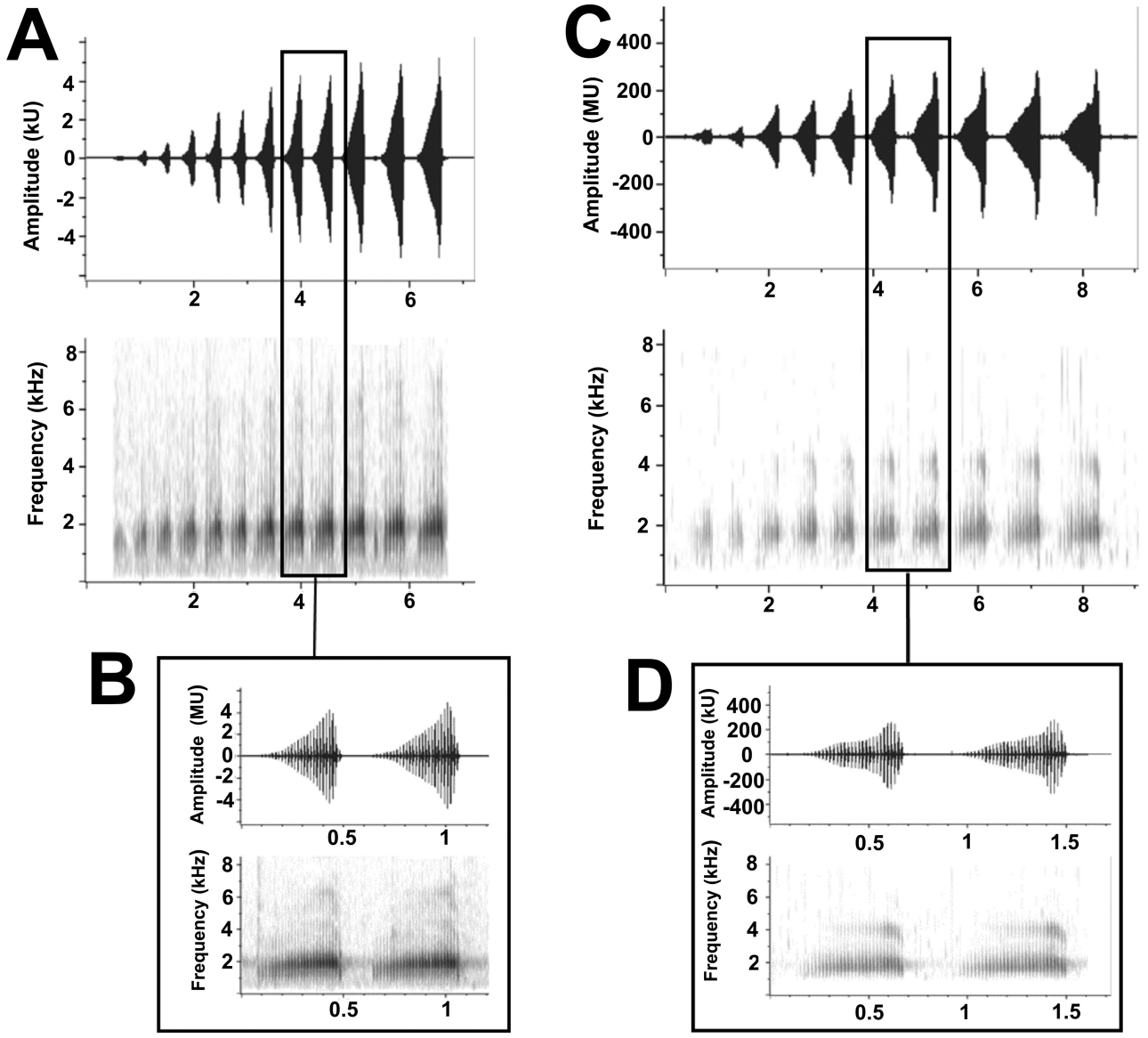

FIGURE 6. Comparison of the male advertisement calls of **A–B)** *L. littlejohni* (ABTC 80813) McKenzie Road, Watagan Mountains, and **C–D)** the holotype of *L. watsoni* **sp. nov.** (AMS R186898), Parma Creek Nature Reserve. **A)** and **C**) Waveform and spectrogram of a single call; **B**) and **D**) Waveform and spectrogram of two notes with the time axis expanded, showing the pulses. Calls in **A**) and **C**) comprise 11 and nine notes respectively.

Conservatively, we treat the northern and southern genetic clusters within *L. watsoni* **sp. nov.** found in the SNP analysis as a single species. First, the molecular data do not provide conclusive evidence that the clusters represent taxa as the mtDNA data do not provide evidence of separate evolutionary lineages and the proportion of variation explained by the divergence between the clusters in the SNP PCoA is quite small. Second, too few samples are presently available from the northern genetic cluster to adequately assess morphological divergence. In our samples, the clusters are allopatric, separated by more than 30 km, further field work to test their genetic isolation in the Ettrema and Jervis subregions of the Sydney Basin Bioregion is needed.

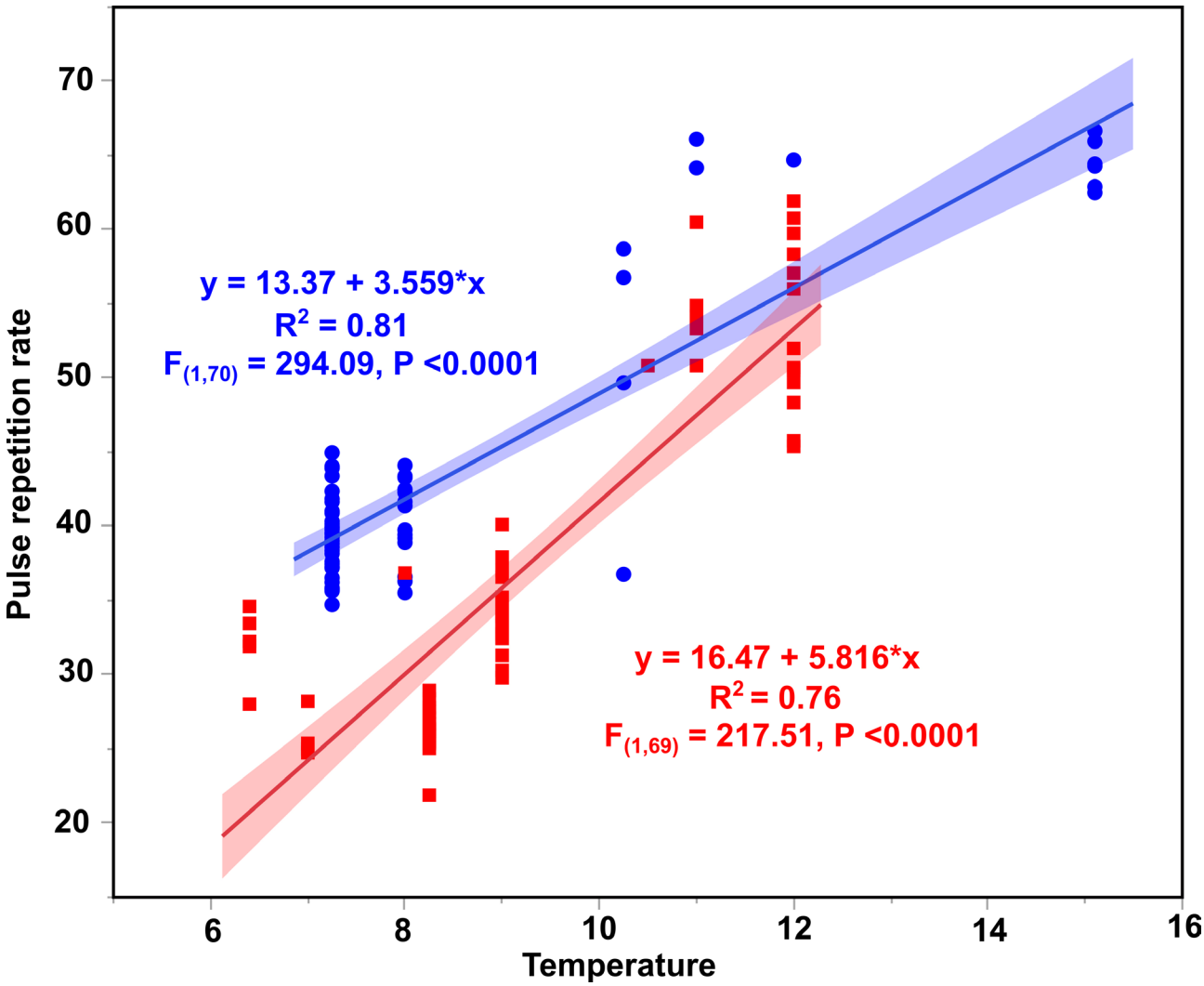

FIGURE 7. Regression of pulse repetition rate against temperature. *Litoria littlejohni*—red circles, *L. watsoni* **sp. nov.**—blue squares. Equations for the regression lines, R^2 value and F test for significance for each species are also included. Shaded areas 90% CI.

Taxonomy

Litoria littlejohni and *L. watsoni* **sp. nov.** share the following features of the *L. ewingii* species group sensu Tyler & Davies (1978): squat small to medium frogs with a maximum length of 35–61mm. The fingers are short, broadly fringed and webbed at least at the base. Moderate to long hindlimbs, toes webbed at least at the base. The dorsum is usually brown or grey, bearing paler or darker, longitudinally orientated stripes. Several species have dark lateral stripes on the head but not extending onto the body.

Tyler & Davies (1978) consider that members of the *L. ewingii* species group are static-water breeders. However, we have observed that, while *L. littlejohni* and *L. watsoni* **sp. nov.** breed in static forest pools, numerous other populations of both species breed in streams, and although breeding and tadpole occupancy may occur mainly in larger connected and sometimes isolated rock pools, the water is not entirely static.

We apply the name *Litoria littlejohni* White, Whitford & Mahony to the northern populations on the basis that we genotyped frogs from the Watagan Mountains near the type locality at Walker's Ridge Road (Joe's Point), Watagan State Forest. We note that the co-ordinates for the type location given in White *et al.* (1994) are incorrect, the correct co-ordinates are -33.067° S, 151.266° E.

White *et al.* (1994) included all museum vouchers available at the time as paratypes of *L. littlejohni* that now include vouchers of both *L. littlejohni* and *L. watsoni* **sp. nov.** We have reassigned the paratypes to the relevant spe-

cies (see Supplementary Table S2). We note however that we were unable to locate specimens from the Canberra College of Advanced Education and therefore we could not examine them, but we can assign them to *L. watsoni* **sp. nov.** based on their collection locations.

Litoria watsoni **sp. nov.**

Holotype. AMS R186898. An adult male collected from Parma Creek, New South Wales (-35.02062° S, 150.4962° E) by Stephen Mahony on 6 September 2016.

Dimensions of holotype (mm). SVL, 50.2; HL 14.0; HW, 17.8; EN, 4.5; IN, 4.1; EYE, 5.2; TYM, 3.0; TBL, 27.1; TAL, 16.7.

Description of the holotype. The body form, colour and pattern of the holotype are illustrated with an in life image in Fig. 8D. Head longer than wide (HL/HW = 0.79); head widest at eyes; snout rounded in lateral and dorsal profiles. Nostrils prominent in dorsal profile. Single row of vomerine teeth running laterally anterior to choanae. The tympanum is circular and visible, diameter equal to eye diameter (TYM/EYE = 0.58).

Prominent terminal discs on all toes and fingers, no webbing between fingers, and toes with basal webbing. Finger length 3>4>2>1; toe length 4>5=3>2>1. Sub-articular tubercules present under fingers and toes but not prominent. Inner metatarsal tubercule present and prominent, approximately one third of the length of first toe. Nuptial pad dark brown, oval, on dorsal surface only of the proximal half of the first digit. Legs relatively long (TBL/SVL = 0.54).

Skin texture of back weakly granular, becoming more granular laterally and on the venter and thighs. Ventral surface granular. Upper surfaces of legs and arms and lower surfaces of lower legs and arms smooth.

Variation. Summary of variation in morphometric measurements for each sex is presented in Table 3 and appearance in Fig. 8. Male SVL 42–59 mm female SVL 50–64 mm; head length relative to head width variable (HL/HW range 0.66–0.97). The tympanum diameter is variable in size relative to eye length (TYM/EYE range 0.44–0.72). Legs relatively long (TBL/SVL 0.44–0.59).

Color in life. Dorsal surfaces of body and limbs light brown mottled with dark and lighter flecking of brown and yellow. The side of the face and extending back beneath the tympanum to the axil is a lighter shade of the dorsal colouring (Fig. 9). Colour on back of upper and lower leg and onto the foot, groin and posterior flanks, and on the upper axil of the forelimb is an immaculate reddish-orange wash (Fig. 9). A darker brown to black line extends from the external nostril along the canthus rostralis to the eye, continuing less intensely behind the eye over the tympanum and then onto the flank where it gradually dissipates. Ventral surface white, with the exception of the upper legs which have an orange wash. The gular region has a yellowish wash. Iris is yellowish gold.

Advertisement call. This description of the call is based on eleven individuals from four locations (Supplementary Table S1, Table 6). The call is a series of moderately strident notes sounding like "wriiik..wriiik..wriik…wriik" which increase in volume to the last note, and is placed in the "pulse repetition sound" category of Beeman (1998). The call is moderately long (mean 5.6 s), comprising (mean 6.5, range 3 to 14) repeated notes of short duration (mean 0.58 s) each separated by a shorter interval (0.40 s). The repeated notes increase in amplitude across the call, and each consists of distinct pulses (Table 6, Fig. 6). Note duration and inter-note duration also increases gradually across the call and is accompanied by a gradual increase in the number of pulses (mean 22.8, range 7 to 33) in each note. The note envelope is fully amplitude modulated with distinct short pulses separated by a duration about three times longer, with the amplitude rising to a peak followed by a rapid decay (Fig. 6). Spectrally, there is no evidence of frequency modulation in the call or in the notes. Dominant frequency of the call has a mean of 1740 Hz (range 1505 to 2018 Hz).

Etymology. Named in honour of Dr Graeme Watson, formerly of the University of Melbourne, Victoria, Australia, for his lifelong contribution to the ecology and evolutionary biology of Australian amphibians and his particular contribution to elucidating the evolutionary relationships in the *L. ewingii* species group.

Distribution. Found in eastern Victoria and south-eastern New South Wales, from 10 km east of Bellbird Creek and 4 km south of Brookville in eastern Victoria, along the eastern fall of the Great Dividing Range north to the Budderoo Plateau, Illawarra Region, NSW.

Altitudinally, *L watsoni* **sp. nov.** occurs from near sea level, e.g. Parma Creek (179 m asl) and Nadgee State Forest (198 m a.s.l.) to about 1,100 m a.s.l. in the upper reaches of the Mongarlowe River, near Braidwood, NSW.

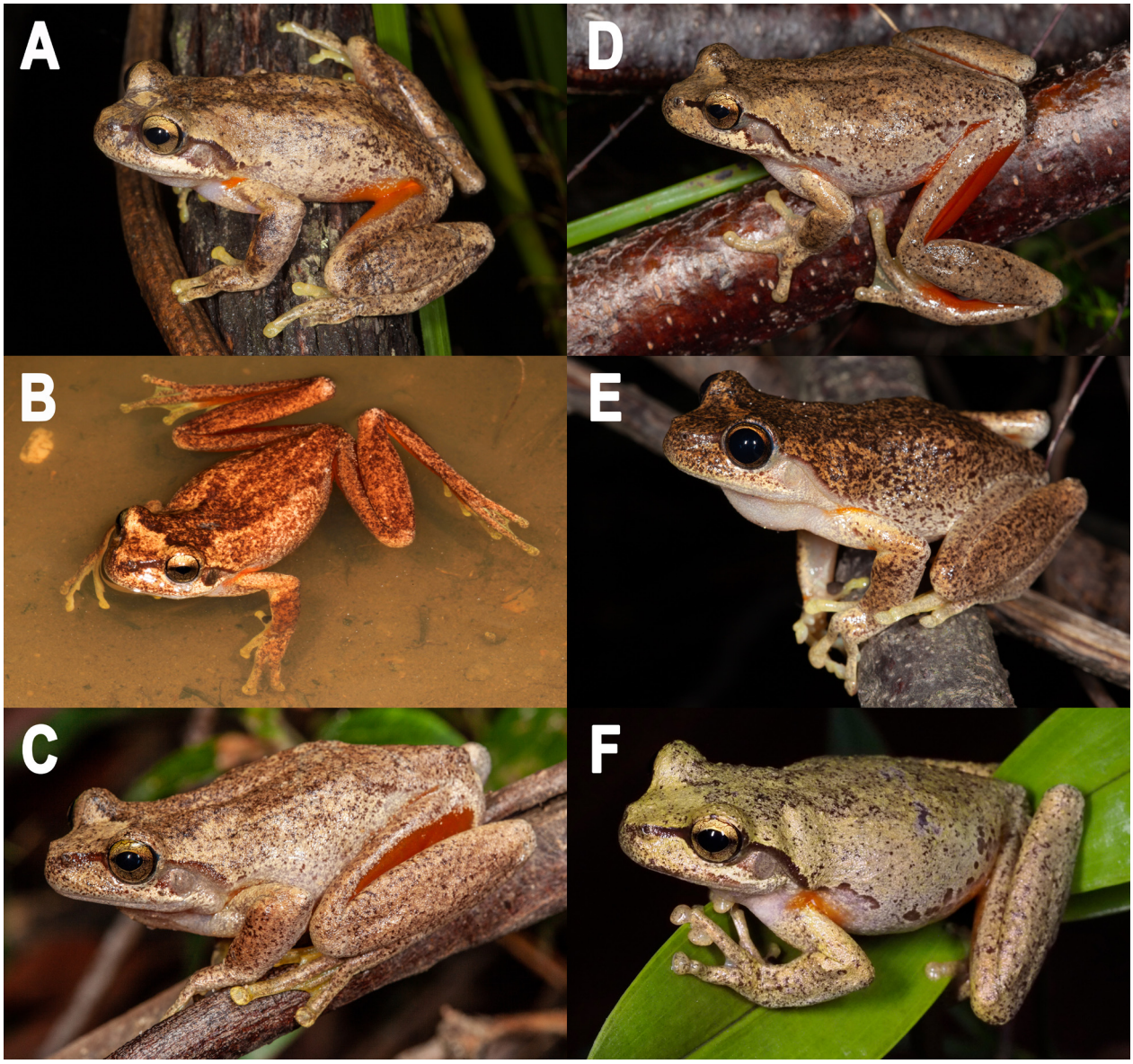

FIGURE 8. Images in life of *Litoria littlejohni* **A–C**) unvouchered males Sawmill Pond, Watagan Mountains, NSW; and *Litoria watsoni* **sp. nov.**, **D)** holotype, adult male (AMS R186898) Parma Creek Nature Reserve, NSW, **E)** adult male Parma Creek Nature Reserve, NSW, **F)** adult male Gerringong Falls, Budderoo Plateau, NSW.

The ranges of *Litoria watsoni* **sp. nov.** and *L. littlejohni* appear to abut at the southern boundary of the Sydney Basin Bioregion. The southern end of the Sydney Basin Bioregion is characterised by ranges of sedimentary sandstones and silts that are deeply dissected by rivers forming steep escarpments and v-shaped valleys, and has been recognised as a biogeographic barrier for several taxa (Bryant & Krosch 2016). However it does not appear to be associated with a distinct drier and warmer landscape change as is the case for other biogeographical barriers in north-eastern Australia such as the St Lawrence and Burdekin gaps. Additional research is required at the southern border of the Sydney Basin Bioregion and the northern border of the South East Corner Bioregion to better understand the distribution of *L. littlejohni* and *L. watsoni* **sp. nov.** to determine whether they are sympatric in the zone where these two bioregions meet.

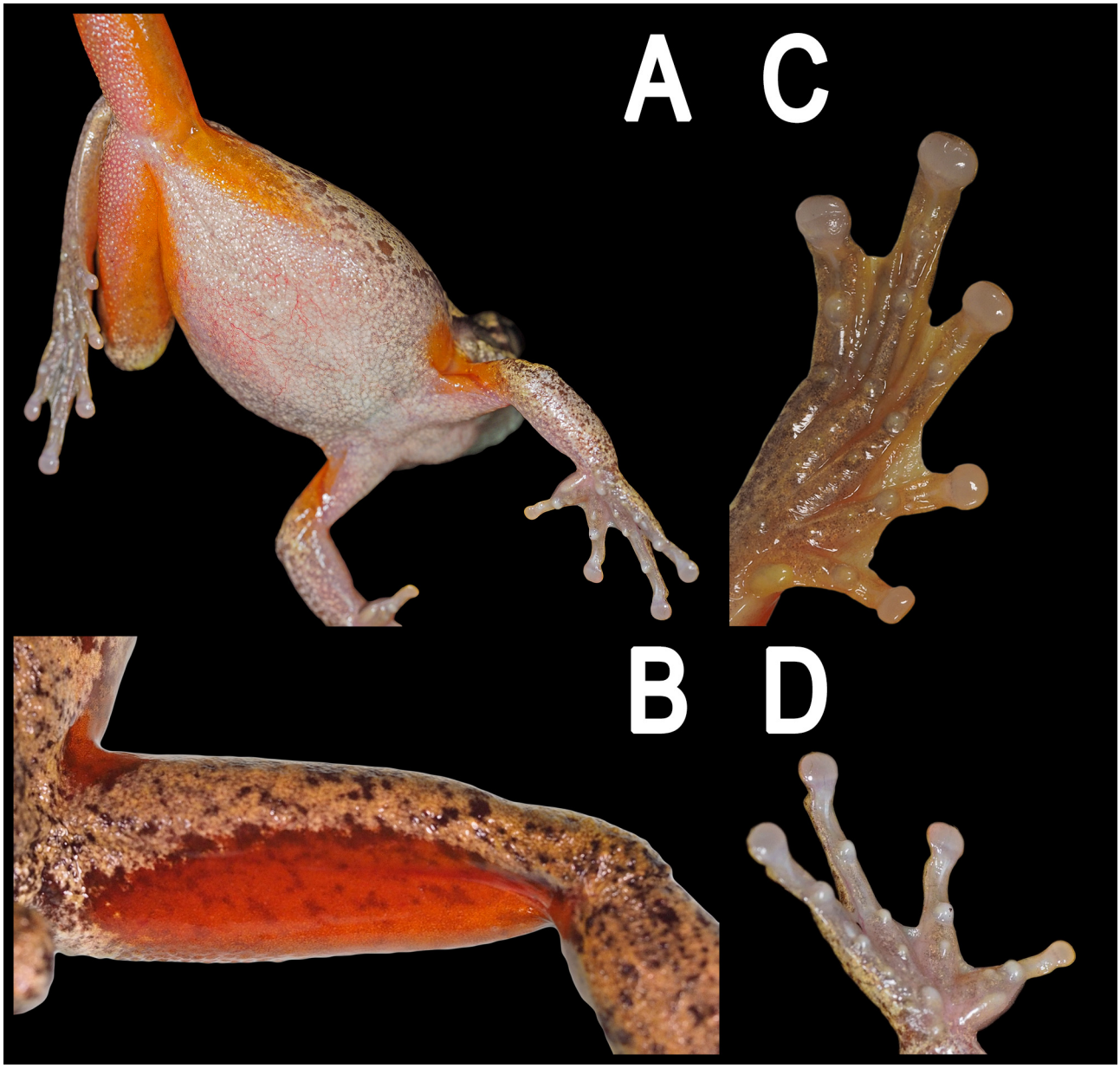

FIGURE 9. Images of *Litoria watsoni* **sp. nov.** in life from the Parma Creek Nature Reserve and Budderoo Plateau, NSW. **A**) ventral view showing colour under legs and in axilla, **B**) back of thigh, **C**) plantar view of foot, **D**) palmar view of hand.

Habitat. *Litoria watsoni* **sp. nov.** occurs in several different vegetation communities including numerous sites of post-forest harvest regrowth (Daly & Craven 2007, Lemckert 2010, Gillespie *et al.* 2016). At the northern extent of its distribution in the Shoalhaven River catchment (Parma Creek Nature Reserve and Barren Grounds Nature Reserve on the Budderoo Plateau), where it appears to be most abundant, it is generally associated with upland heath and dry sclerophyll forest communities. In southern NSW and eastern Victoria records are generally in wet forests (Gillespie *et al*. 2016), although there are two historic records in heath in Nadgee Nature Reserve in south-eastern NSW (ALA accessed June 2020). Where *L. watsoni* **sp. nov.** is associated with heathland, the soils are sandy, and the parent geology is sandstone. In these locations the breeding sites are in streams that flow slowly across a mostly horizontal bedding plane on a plateau or steppe, and are not found in the v-shaped valleys or coastal valleys that form once the streams descend from the plateau (Fig. 10). Dense heath vegetation, comprising various species of *Banksia* and *Grevillia*, border and overhang the streams, and males typically call from branches up to a 1.5 m above the stream or from deep within large clumps of ferns. Preferred streams are shallow, characterized by rocky or sandy bases, potholed and with lateral rocks bars creating pools that are either completely isolated from surrounding water bodies, or larger connected pools (Fig. 10). Where associated with wet forests communities the field records cite

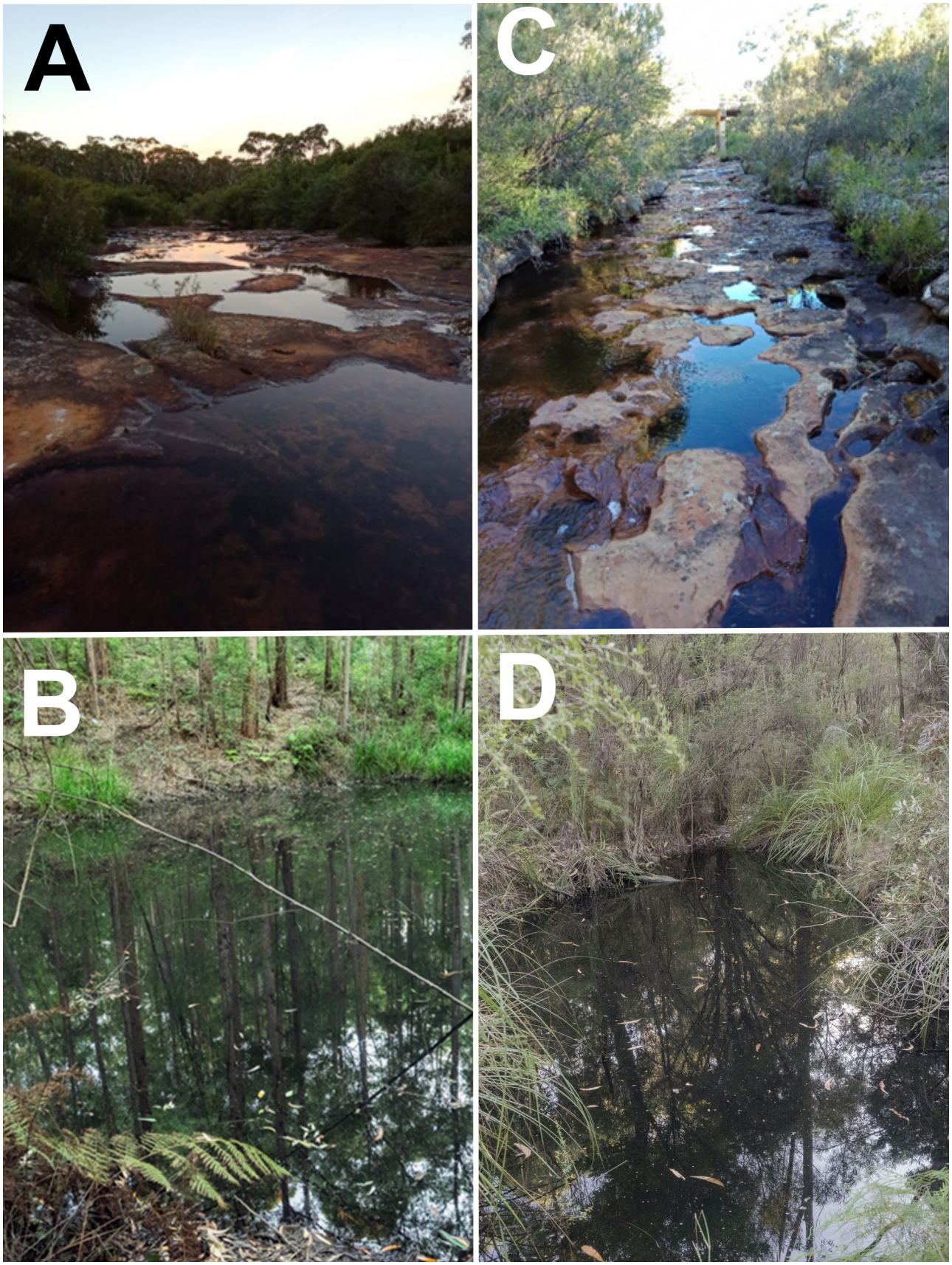

FIGURE 10. Photographs of breeding habitat for *L. watsoni* **sp. nov. A**) Permanent stream with larger interconnected and isolated pools at Tianjara (*K. Klop*-Toker), **B**) pond in Nadgee (R. Bilney); and *L. littlejohni* **C**) permanent stream with larger interconnected and isolated pools at Dharawal National Park (*K. Klop*-Toker), **D**) pond in the Watagan Mountains.

roadside ditches and puddles in south-eastern NSW and eastern Victoria (Littlejohn & Watson 1966). Whether *L. watsoni* **sp. nov.** prefers these landscape features, or is apparently absent from larger more permanent water bodies and streams due to field survey design, is difficult to determine from available data. We know of no records in heavily modified landscapes such as farmlands or urban areas, and there is some debate about whether the species is affected by forestry practices (Lemckert 2004, 2010; Gillespie *et al.* 2016).

Breeding Biology. Little is known about the larval ecology of *L. watsoni* **sp. nov.** and studies are a matter of priority given its threatened conservation status (see below).

Males call along permanent streams in all seasons of the year particularly following rainfall (Gillespie *et al.* 2016). Males have been observed calling at a small forest pond at Nadgee and Yadboro State Forest in far south-eastern NSW after summer rain (Bilney pers. obs.). Calling behaviour by *L. littlejohni* in relation to season and permanent streams and ephemeral habitats is very similar to that of *L. watsoni* **sp. nov.** (Lemckert & Mahony 2008, Lemckert 2010, Mahony 1993, White *et al.* 1994).

In a composite description based on samples from the Cann River, Victoria, the Mongarlowe River, NSW and Budderoo Plateau, NSW (referable to *L. watsoni* **sp. nov.**) and from the Watagan Mountains, NSW (referable to *L. littlejohni*), Anstis (2017) described oviposition sites, egg mass structure, and embryo morphology, without noting any differences in these attributes among the sampled locations. Martin & Littlejohn (1966) described the larvae of *L. watsoni* **sp. nov.** from Cann River, Victoria (as *L. jervisiensis*).

Diagnosis. *Litoria watsoni* **sp. nov.** can be distinguished from all other members of the *L. ewingii* species group except *L. littlejohni* by the occurrence of immaculate orange markings on the anterior and posterior surfaces of the femur and tibia, in the groin and posterior flanks, and by its larger size (see Anstis [2017] for comparative measurements). The call can be distinguished from that of *L. littlejohni* by the lower number of pulses in each note (mean 22.8 compared to 27.8) (Table 4). From a genetic perspective, apomorphic nucleotide states at 21 sites in the mitochondrial *ND4* gene reliably diagnose *L. watsoni* **sp. nov.** from *L. littlejohni* (Table 3).

Conservation assessments for *L. littlejohni* and *L. watsoni* sp. nov.

The Atlas of Living Australia, as of 20 April 2020, has a total of 2411 records, 2064 that are referrable to *L. littlejohni* and 347 to *L. watsoni* **sp. nov**. The AOO of *L. watsoni* **sp. nov.** is 308 km^2, and for *L. littlejohni* it is 390 km^2. The AOO for both species are below the IUCN threshold level of 500 km^2 for species with evidence of continued declines and which are severely fragmented. These estimates result in the classification for both species of "Endangered" [IUCN Red List Assessment: Criteria 1. A.2(a)(c)(e), and Criteria 2. B.2 (a),(b)(i)(ii)].

Both *L. littlejohni* and *L. watsoni* **sp. nov.** have patchy, but widespread distributions, and this would tend to indicate that they are habitat specialists, and that the preferred habitat is itself patchy. However, no habitat specialisation has been identified in descriptive studies (Daly & Craven 2007), or from modelling approaches (Lemckert 2010, Lemckert & Mahony 2010).

In contrast to the low AOO values, the EOO for both species is relatively large, i.e., *L. watsoni* **sp. nov.** is 31,950 km^2 and *L. littlejohni* is 16,317 km^2. The larger values serve to demonstrate the extent of spatial fragmentation of the historical records for both species (Fig. 11). They also indicate that there are large areas of potential habitat where the species have not been recorded. The distribution of both species includes large areas of wilderness and habitats that are not subject to regular ecological monitoring. *Litoria littlejohni* has historic records in the Blue Mountains World Heritage Area, a vast area of 1.03 million hectares of sandstone plateau, escarpment and gorges (UNESCO 2020), of mostly natural vegetation, much of which is accessible only by foot. Similarly, there are large areas of potential habitat for *L. watsoni* **sp. nov.** in several National Parks on the eastern fall of the Great Dividing Range in south east NSW and in eastern Gippsland, Victoria. For example, Morton National Park at the northern extent of the range of *L. watsoni* **sp. nov.** is an area of 1,997 km^2 with numerous plateaus and streams with habitats similar to that described above.

Despite these large natural areas, there is compelling evidence of declines and disappearances of populations of both species in the past 30 years (Fig. 11). In east Gippsland *L. watsoni* **sp. nov.** was observed at only six sites between 2009 and 2015 despite targeted surveys (Gillespie *et al.* 2016). Similarly, we are aware of only three observations in far south-eastern NSW between 2010 and 2020 despite more than 100 hours of targeted field surveys and the use of acoustic recorders for over 12 months at eight historic locations (Moses & Mahony unpubl.) (Fig. 11).

Absence of observations are not explained comfortably by the rugged nature and isolation of the areas. Both regions have large areas of native hardwood forests that are selectively logged with some native hardwood plantations. Surveys are undertaken by forestry industry ecologists, and there have been two doctoral research projects that have investigated forest dwelling and stream associated amphibians (*Heleioporus australiacus* and *Litoria spenceri*), in portions of these areas, without observing populations of *L. watsoni* **sp. nov.** (Gillespie & Hollis 1996, Penman *et al*. 2004, Penman 2005). We consider that focused field studies are necessary in the areas where there have been no records in the past three decades, so that appropriate decisions can be made about conservation actions.

Evidence of declines and disappearance of *L. littlejohni* populations come from within large areas of land managed for nature conservation and as water catchments where public access is minimal. The Greater Blue Mountain National Park and World Heritage Area covers a considerable portion of sandstone plateau landscape known to provide preferred habitat for this frog within the Sydney Basin Bioregion, and we are aware of only three recorded populations in this area in the past decade (Fig. 11).

At the north-eastern extremity of the Sydney Basin Bioregion, the Watagan Mountains supports a small population of *L. littlejohni* (Fig. 11). In this area it is also associated with first order streams on a small sandstone plateau, and with numerous forest pools constructed as part of forestry operations. There is evidence of a small decline in occupancy in this area (Mahony 1993), but the species has been observed in natural and forestry modified habitats over a twenty-year period (Lemckert 2011). Populations are not large and choruses comprise two to six males. Threats in this area relate mostly to human impacts such as damage from recreational vehicles.

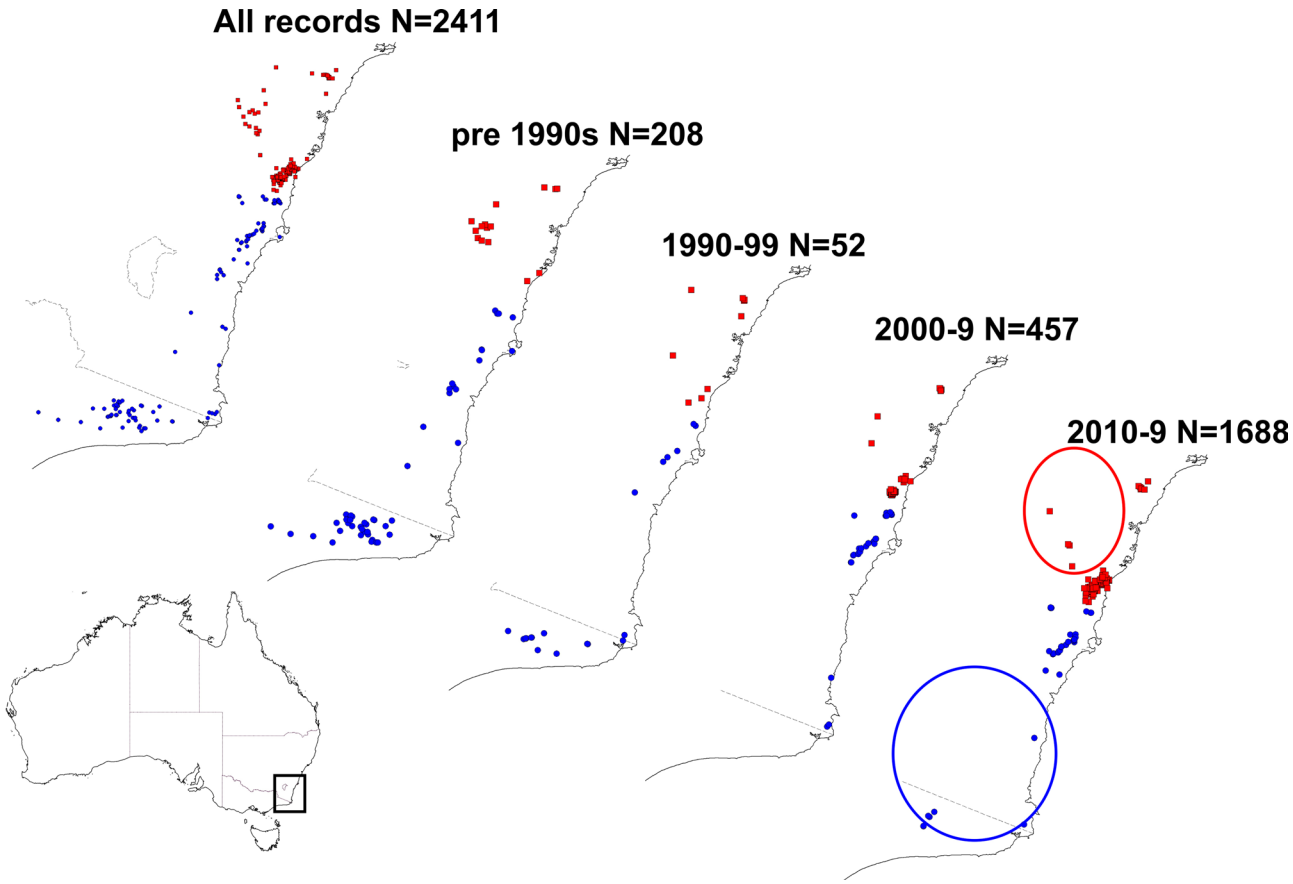

FIGURE 11. Historic distribution of *L. littlejohni* and *L. watsoni* **sp. nov.** by decadal sampling periods from the ALA (accessed May 2020). Circles show the areas of the far south east of the distribution of *L. watsoni* **sp. nov.** and in the Greater Blue Mountains area in the north west of the distribution of *L. littlejohni* where records have declined.

In contrast to the situation for *L. watsoni* **sp. nov.** and most of the range of *L. littlejohni,* the Woronora Plateau area has supported a stable population over the last 20 years. Since 2002, *L. littlejohni* has been recorded on the Woronora Plateau in a series of intensive surveys by mining consultants and government (DECC 2007, present study). This plateau which has several catchments that are dammed as part of the water supply for the metropolitan area of Sydney, is protected and is covered with native vegetation. There are over 1940 individual records of the

frog from this area in the Atlas of Living Australia, which represent records from first order streams from four river catchments that flow over a sandstone topped plateau with subdued relief. Many of the records from this area are repeated surveys along the same streams, and do not represent newly reported populations (Fig. 11), and the total AOO for this area is 216 km^2. The natural values of the surface habitats are protected in the Sydney Water Catchment Area, however there is a threat to the streams from land subsidence and cracking of the surface rocks and creek bases due to underground mining in several of the catchments that provide habitat for the frog (DECC 2007).

The amphibian disease chytridiomycosis may be responsible for the observed declines. Although we do not have pre-decline demographic data, there is evidence that chytrid occurs on other frog species found within the geographic range of both *L. littlejohni* and *L. watsoni* **sp. nov.** (Kriger *et al.* 2007), and population losses over the past three decades coincide with the known occurrence and spread of the chytrid pathogen in eastern Australia (Berger & Speare 1998, Skerratt *et al.* 2007, Mahony *et al*. 2013). Furthermore, chytrid infected *L. littlejohni* have been found on the Woronora Plateau (DECC 2007). Both species are likely to be susceptible to chytridiomycosis, and urgent conservation actions are necessary to prevent the loss of isolated populations and the genetic diversity they represent. Studies are needed urgently to understand why a few populations remain robust, and whether this is because they are resistant to chytrid, or occur in habitats that provide some protection, or where chytrid does not occur presently (Scheele *et al*. 2014b).

Discussion

While the temporal patterns of distribution and abundance of *L. littlejohni* and *L. watsoni* **sp. nov.** are consistent with declines due to a relatively recent threatening process, the relative rarity of both species can confound such an interpretation. Therefore, it is important to identity the reasons why a species is rare so that the appropriate conservation actions can be developed. Rarity may be the result of long-term evolutionary processes and be a natural feature, or it may be the outcome of specific threats that are related to human activities.

A species may be apparently rare because of a number of factors—it maybe a habitat specialist and its habitat is restricted, or it has cryptic habits and detection probability is low, or there is insufficient survey effort, or it has experienced extensive declines due to anthropogenic landscape impacts, e.g. land clearing or disease (Knapp 2011). Here we explore each of these four factors for *L. littlejohni* and *L. watsoni* **sp. nov.**

First, several studies have investigated the habitat requirements of *L. littlejohni*, and *L. watsoni* **sp. nov.** (as *L. littlejohni*) to test whether it is a habitat specialist which may explain its rarity (Daly & Craven 2007, Lemckert 2010, Gillespie *et al.* 2016). There are fewer than 30 extant populations known for both species combined across a relatively long but narrow distribution that spans 5 degrees of latitude (about 800 km) and at its maximum is only 120 km wide, from central-eastern New South Wales south to north-eastern Victoria. They typically occur at mid to higher elevations, on the eastern fall of the Great Dividing Range (Daly & Craven 2007), and have not been recorded from the coastal plain or coastal swamps and heaths, or from cleared agricultural land. They are found only in native vegetation communities, but they can vary from dense upland heaths to dry and wet sclerophyll eucalypt forests (White *et al.* 1994, Daly & Craven 2007, Lemckert 2010, Gillespie *et al.* 2016). Their ranges are coincident with large areas of native vegetation, mostly eucalypt forest and heath communities, with about equal amounts in areas where forestry occurs and lands in conservation reserves. Several lower elevation riverine valleys that have been cleared for agriculture may once have provided suitable habitat. We know of no population of either species outside of forested habitats. Lemckert (2010) found that occupied habitat of *L. littlejohni* was associated with a lack of grass in the understory, higher moisture environments, and flatter areas (negative to rough terrain), features that are widespread in the native forests. Together these habitat features do not indicate that *L. littlejohni* is a habitat specialist, and when combined with observations of breeding habitat (Daly & Craven 2007), and vegetation associations, do not provide a clear understanding of why this species is observed so rarely. To emphasize the potential generalist nature of both species, the habitats used for breeding includes lotic and lentic situations. To the south of the Sydney Basin bioregion, *L. watsoni* **sp. nov.** breeds in slow moving permanent streams that pass over sandstone substrates (Daly & Craven 2007), and in north-eastern Gippsland and southern NSW in the South East Corner bioregion, it breeds in ephemeral pools with clay or sandy substrates (Gillespie *et al*. 2016; this study). *Litoria littlejohni* breeds in ephemeral and permanent ponds on clay and sandy substrates and in slow moving permanent streams on sandstone (Mahony 1993, White *et al*. 1994). In summary the ecological information indicates they are generalist species, and that their habitats are not restricted.

Second, there is no evidence that *L. littlejohni* or *L. watsoni* **sp. nov.** are particularly cryptic in habit that would explain their rarity. Both are relatively large (adult female snout to vent up to 60 mm), with distinctive markings and moderately strong mating call (White *et al*. 1994). They are one of the few species in the *L. ewingi* species group where breeding events are episodic following heavy rainfall, which may occur in all seasons including autumn and winter (Mahony 1993, Lemckert & Mahony 2008). It could be argued that they are not regularly reported since most surveys occur in spring and summer, and the probability of detection of adults may be low due to the brief breeding period. However, the larvae are distinctive (Anstis 2017) and often present for several months in ponds and streams, and it would be expected that they would be detected in surveys. However, tadpole surveys have not until recent times been included in recommended field survey protocols (DECC 2010). Therefore, in summary we consider that it is not likely that rarity is an outcome of cryptic behavior leading to poor detection.

Third, it is also not likely that the lack of recorded observations is due to a lack of survey effort. This is not a suitable explanation since the forestry estate is distributed across the range and is subject to ecological surveys. Although these do not occur over a large spatial scale in any one year, over the period of a decade a large proportion of the area would be surveyed (Penman *et al.* 2005, Lemckert 2011, Gillespie *et al.* 2016). A similar observation can be made for lands under conservation management (DEC 2007, Gillespie *et al.* 2016).

Fourth, a possible explanation for rarity is that populations have declined, and there are several potential causes for the widespread decline of forest dependent habitat generalists. Declines may be associated with human activities such as land clearing or forestry harvesting (Gillespie *et al*. 2016). Although all records are within native vegetation communities, which might imply that the species does not cope well with disturbance, several sites are within post-harvest forest regrowth (Daly & Craven 2007; Lemckert 2004, 2010; Gillespie *et al.* 2016). Furthermore, population disappearances have occurred in several long-established national parks where forestry practice and habitat disturbance are not feasible reasons for population declines.

A more plausible explanation for the declines in both species is the disease chytridiomycosis that is caused by the introduced amphibian pathogen *Batrochochytrium dendrobatidis* (chytrid). Most surveys that could have detected *L. littlejohni* and *L. watsoni* **sp. nov.** were conducted since the arrival of the chytrid fungus in Australia in the mid-1970s (Berger & Speare 1998), and the signal of decline may not be detected by post decline surveys. Members of the *L. ewingii* species group, of which *L. littlejohni* and *L. watsoni* **sp. nov.** are members, are distributed in the cooler mesic zone of Australia where chytrid impacts have been widespread (Scheele *et al.* 2014a, Brannelly *et al*. 2016). Another member of this species group, *L. verreauxii alpina*, is impacted greatly by chytridiomycosis with marked declines in distribution and abundance (Scheele *et al.* 2014a). It seems entirely plausible that *L. littlejohni* and *L. watsoni* **sp. nov.** have gradually declined from large areas of their former distribution due to impacts of the pathogen. However, direct evidence does not exist as we are not aware of any reports of moribund individuals or studies that have tested whether the species is susceptible to chytridiomycosis or any field surveys that have investigated whether the species is infected by chytrid.

Evidence of declines comes from examination of the Atlas databases which shows that in the last decade there is only one region where *L. littlejohni* show consistent reports of the species presence, on the Woronora Plateau on the south-eastern margin of the Sydney Basin Bioregion (Atlas of Living Australia, BioNet, Victorian Biodiversity Atlas, DEC 2007, Daly & Craven 2007, Gillespie *et al*. 2016). Repeated annual streams surveys in this area show that the population is relatively large and robust (ALA database records). Most of this area is within protected lands such as the South Western Sydney Water catchment area, a National Park and a Nature Reserve. However, the species has not been found in large tracts of contiguous lands to the west and south of this area, much of which is conservation reserves. If our proposal that the decline of populations of this species is due to chytrid is correct, the apparent robust nature of population on the Woronora Plateau may prove to be important in understanding specific environmental niches or intrinsic biological factors such as genetically determined immunity that enable populations to persist in the face of chytridiomycosis (Puschendorf *et al.* 2013).

Acknowledgements

We thank G. Gillespie, M. Schulz, and G. Madani for collecting tissue samples, R. Bilney, J. Mulder, M. Littlejohn and D. Stewart for supplying calls and field site information, K. Klop-Toker and R. Bilney for images; M. Hutchinson (SAMA), J. Rowley (AMS), L. Joseph (ANWC), and Jane Melville (MV) for access to vouchers in their care,

and T. Bradfield, L. Price and S. Catalano for some of the mitochondrial sequencing. The Australian Biological Resources Study (grant number 205-54), and the NSW NPWS Saving Our Species program provided financial support. Fieldwork in NSW by MM, SM and BM was undertaken under Scientific Licence from the NSW Office of Environment and Heritage (National Parks and Wildlife), and under Animal Ethics approval from the University of Newcastle Animal Ethics Committee. Fieldwork in NSW State Forests was conducted under a Special Purpose Permit, and access to field study sites was facilitated by the senior ranges at several National Parks in NSW.

Literature cited

Anstis, M. (2017) *Tadpoles and frogs of Australia*. New Holland Publishers Pty Limited, Chatswood, 848 pp.

Anstis, M., Price, L.C., Roberts, J.D., Catalano, S., Doughty, P., Hines, H.B. & Donnellan, S.C. (2016) Revision of the Australian water holding frog (*Cyclorana platycephala*, Anura: Hylidae), with a description of a new species and subspecies. *Zootaxa*, 4126 (4), 451–479.
https://doi.org/10.11646/zootaxa.4126.4.1

Beeman, K. (1998) Digital signal analysis, editing, and synthesis. *In*: Hopp, S.L., Owren, M.J. & Evans, C.S. (Eds.), *Animal acoustic communication*. Springer, Berlin and Heidelberg, pp. 59–103.
https://doi.org/10.1007/978-3-642-76220-8_3

Berger, L. & Speare, R. (1998) Chytridiomycosis: A new disease of wild and captive amphibians. *ANZCCART Newsletter*, 11, 1–3.

Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., Ragan, M.A., Hyatt, A.D., McDonald, K.R., Hines, H.B., Lips, K.R., Marantelli, G. & Parkes, H. (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 9031–9036.
https://doi.org/10.1073/pnas.95.15.9031

Brannelly, L.A., Hunter, D., Skerratt, L.F., Scheele, B., Lenger, D., McFadden, M.S., Harlow, P.S. & Berger, L. (2016) Chytrid infection and post-release fitness in the reintroduction of an endangered alpine tree frog. *Animal Conservation*, 19, 153–162.
https://doi.org/10.1111/acv.12230

Bryant, L.M. & Krosch, M.N. (2016) Lines in the land: a review of evidence for eastern Australia's major biogeographical barriers to closed forest taxa. *Biological Journal of the Linnean Society*, 119, 238–264.
https://doi.org/10.1111/bij.12821

Chifman, J. & Kubatko, L. (2014) Quartet inference from SNP data under the coalescent model. *Bioinformatics*, 30, 3317–3324.
https://doi.org/10.1093/bioinformatics/btu530

Courtois, B., Audebert, A., Dardou, A., Roques, S., Ghneim- Herrera, T., Droc, G., Frouin, J., Rouan, L., Gozé, E., Kilian, A., Ahmadi, N. & Dingkuhn, M. (2013) Genome-wide association mapping of root traits in a Japonica rice panel. *PLoS ONE*, 11, e78037.
https://doi.org/10.1371/journal.pone.0078037

Cruz, V.M.V., Kilian, A. & Dierig, D.A. (2013) Development of DArT marker platforms and genetic diversity assessment of the U.S. collection of the new oilseed crop Lesquerella and related species. *PLoS ONE*, 8, e64062.
https://doi.org/10.1371/journal.pone.0064062

Daly, G. & Craven, P. (2007) Monitoring populations of the Heath Frog *Litoria littlejohni* in the Shoalhaven region on the south coast of New South Wales. *Australian Zoologist*, 34**,** 165–172.
https://doi.org/10.7882/AZ.2007.014

DECC (2007) Terrestrial Vertebrate Fauna of the Greater Southern Sydney Region: Volume 5—The Fauna of the Blue Mountains Special Areas. *A joint project between the Sydney Catchment Authority and the Department of Environment and Climate Change (NSW) (DECC) under the Special Areas Strategic Plan of Management (SASPoM) by the Information and Assessment Section, Metropolitan Branch, Climate Change and Environment Protection Group, DECC, Hurstville*, Project No: RD01, 1–242.

Donnellan, S.C. & Aplin. K.P. (1989). Resolution of cryptic species in the New Guinean lizard *Sphenomorphus jobiensis* (Scincidae) by electrophoresis. *Copeia*, 1989, 81–88.
https://doi.org/10.2307/1445608

Donnellan, S.C., Adams, M., Hutchinson, M. & Baverstock, P.R. (1993). The identification of cryptic species in the Australian herpetofauna—a high research priority. *In*: Lunney, D. & Ayers, D. (Eds.), *Herpetology in Australia: a diverse discipline.* Royal Zoological Society of New South Wales, Mosman, New South Wales, pp. 121–126.
https://doi.org/10.7882/RZSNSW.1993.018

Earl, D. A. (2012). Structure harvester: A website and program for visualizing structure output and implementing the Evanno method. *Conservation Genetics Resources*, 4, 359–361.
https://doi.org/10.1007/s12686-011-9548-7

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32, 1792–1797.
https://doi.org/10.1093/nar/gkh340

Elshire, R.J., Glaubitz, J.C., Sun, Q., Poland, J.A., Kawamoto, K., Buckler, E.S. & Mitchell, S.E. (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS ONE*, 6, e19379.
https://doi.org/10.1371/journal.pone.0019379

Evanno, G., Regnaut, S. & Goudet, J. (2005) Detecting the number of clusters of individuals using the software structure: A simulation study. *Molecular Ecology*, 14, 2611–2620.
https://doi.org/10.1111/j.1365-294X.2005.02553.x

Georges, A., Gruber, B., Pauly, G.B., White, D., Adams, M., Young, M.J., Kilian, A., Zhang, X., Shaffer, H.B. & Unmack, P.J. (2018) Genome wide SNP markers breathe new life into phylogeography and species delimitation for the problematic short-necked turtles (Chelidae: *Emydura)* of eastern Australia. *Molecular Ecology*, 27, 5195–5213.
https://doi.org/10.1111/mec.14925

Gergus, E.W.A., Sullivan, B.K. & Malmos, K.B. (1997) Call variation in the *Bufo microscaphus* complex: implications for species boundaries and the evolution of mate recognition. *Ethology*, 103, 979–989.
https://doi.org/10.1111/j.1439-0310.1997.tb00140.x

Gerhardt, H.C. (1994) The evolution of vocalization in frogs and toads. *Annual Reviews of Ecology and Systematics*, 25, 293–324.
https://doi.org/10.1146/annurev.es.25.110194.001453

Gerhardt, H.C. & Huber, F. (2002) *Acoustic communication in insects and anurans: common problems and diverse solutions*. University of Chicago Press, Chicago, Illinois, 531 pp.

Gillespie, G. & Hollis, G. (1996) Distribution and habitat of the spotted tree-frog, *Litoria spenceri* Dubois (Anura: Hylidae), and an assessment of potential causes of population declines. *Wildlife Research*, 23, 49–75.
https://doi.org/10.1071/WR9960049

Gillespie, G.R., McNabb, E. & Gaborov, R. (2016) The biology and status of the Large Brown Tree Frog *Litoria littlejohni* in Victoria. *Victorian Naturalist*, 133, 128–138.

Grigg, G.C. & Barker, J. (1973) Frog calls of South-eastern Australia. Tape recordings.

Gruber, B., Unmack, P.J., Berry, O.F. & Georges, A. (2018) dartR: An R package to facilitate analysis of SNP data generated from reduced representation genome sequencing. *Molecular Ecology Resources*, 18, 691–699.
https://doi.org/10.1111/1755-0998.12745

Hoang, D.T., Chernomor, O., von Haeseler, A., Minh, B.Q. & Vinh, L.S. (2018) UFBoot2: Improving the ultrafast bootstrap approximation. *Molecular Biology Evolution*, 35, 518–522.
https://doi.org/10.1093/molbev/msx281

IUCN (2012) *Guidelines for application of IUCN Red List criteria at regional and national levels. Version 4.0*. ?????, ?????, ???? pp. [please cite the name of the publisher, city location of the publisher and the total page number]

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler A. & Jermiin, L.S. (2017) ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature Methods*, 14, 587–589.
https://doi.org/10.1038/nmeth.4285

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. & Drummond, A. (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics Applications Note*, 28, 1647–164.
https://doi.org/10.1093/bioinformatics/bts199

Kilian, A., Wenzl, P., Huttner, E., Carling, J., Xia, L., Blois, H., Caig, V., Heller-Uszynska, K., Jaccoud, D., Hopper, C., Aschenbrenner-Kilian, M., Evers, M., Peng, K., Cayla, C., Hok, P. & Uszynski, G. (2012) Diversity arrays technology: A generic genome profiling technology on open platforms. *Methods in Molecular Biology*, 888, 67–89.
https://doi.org/10.1007/978-1-61779-870-2_5

Knapp, S. (2011) Rarity, species richness, and the threat of extinction—are plants the same as animals? *PLoS Biology*, 9, e1001067.
https://doi.org/10.1371/journal.pbio.1001067

Kumar, S., Stecher, G. & Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33, 1870–1874.
https://doi.org/10.1093/molbev/msw054

Köhler, J., Jansen, M., Rodríguez, A., Kok, P., Toledo, L., Emmrich, M., Glaw, F., Haddad, C., Rödel, M. & Vences, M. (2017) The use of bioacoustics in anuran taxonomy: theory, terminology, methods and recommendations for best practice. *Zootaxa*, 4251 (1), 1–124.
https://doi.org/10.11646/zootaxa.4251.1.1

Kopelman, N.M., Mayzel, J., Jakobsson, M., Rosenberg, N.A. & Mayrose, I. (2015) CLUMPAK: a program for identifying clustering modes and packaging population structure inferences across K. *Molecular Ecology Resources*, 15, 1179–1191.
https://doi.org/10.1111/1755-0998.12387

Kriger, K.M., Pereoglou, F. & Hero, J.M. (2007) Latitudinal variation in the prevalence and intensity of chytrid (*Batrachochytrium dendrobatidis*) infection in eastern Australia. *Conservation Biology*, 21, 1280–1290.

https://doi.org/10.1111/j.1523-1739.2007.00777.x
Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T. & Calcott, B. (2017) PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution*, 34, 772–773.
https://doi.org/10.1093/molbev/msw260
Leaché, A.D., Banbury, B.L., Felsenstein, J., de Oca, A.N. & Stamatakis, A. (2015) Short tree, long tree, right tree, wrong tree: new acquisition bias corrections for inferring SNP phylogenies. *Systematic Biology*, 64, 1032–1047.
https://doi.org/10.1093/sysbio/syv053
Lemckert, F. (2004) The biology and conservation status of the heath frog *Litoria littlejohni. Herpetofauna,* 34, 99–104.
Lemckert, F. (2010) Habitat relationships and presence of the threatened heath frog *Litoria littlejohni* (Anura: Hylidae) in central New South Wales, Australia. *Endangered Species Research*, 11, 271–278.
https://doi.org/10.3354/esr00277
Lemckert, F.L. (2011) Managing pond-breeding anurans in the selectively harvested forests of coastal New South Wales, Australia. *Forest Ecology and Management*, 262, 1199–1204.
https://doi.org/10.1016/j.foreco.2011.06.014
Lemckert, F. & Mahony, M. (2008) Core calling periods of the frogs of temperate New South Wales, Australia. *Herpetological Conservation and Biology*, 3, 71–76.
Lemckert, F. & Mahony, M. (2010) The relationship among multiple-scale habitat variables and pond use by anurans in northern New South Wales, Australia. *Herpetological Conservation Biology*, 5, 537–547.
Littlejohn, M. (1965) Premating isolation in the *Hyla ewingii* complex (Anura: Hylidae). *Evolution*, 19, 234–243.
https://doi.org/10.1111/j.1558-5646.1965.tb01709.x
Littlejohn, M. & Martin, A. (1967) The breeding biology and larval development of *Litoria jervisiensis* (Anura: Hylidae). *Proceedings of the Linnean Society New South Wales*, 91, 410–412.
Littlejohn, M. (1976) The *Litoria ewingii* complex (Anura: Hylidae) in south-eastern Australia IV. Variation in mating-call structure across a narrow hybrid zone between *L. ewingii* and *L. paraewingi*. *Australian Journal of Zoology*, 24, 283–293.
https://doi.org/10.1071/ZO9760283
Littlejohn, M. (1971) A reappraisal of mating call differentiation in *Hyla cadaverina* (= *Hyla californiae*) and *Hyla regilla*. *Evolution*, 25, 98–112.
https://doi.org/10.2307/2406502
Littlejohn, M. (1987) *Calls of Victorian Frogs*. Department of Zoology, University of Melbourne, Melbourne, ????? pp. [please cite the total page number]
Littlejohn, M. & Loftus-Hills, J.J. (1968) An experimental evaluation of premating isolation in the *Hyla ewingit* complex (Anura: Hylidae). *Evolution*, 22, 659–663.
https://doi.org/10.1111/j.1558-5646.1968.tb03467.x
Littlejohn, M.J. & Watson, G.F. (1985) Hybrid zones and homogamy in Australian frogs. *Annual Review of Ecology and Systematics*, 16, 85–112.
https://doi.org/10.1146/annurev.es.16.110185.000505
Loftus-Hills, J.J. & Littlejohn, M.J. (1971) Pulse repetition rate as the basis for mating call discrimination by two sympatric species of *Hyla*. *Copeia*, 1971, 154–156.
https://doi.org/10.2307/1441612
Lleonart, J., Salat, J. & Torres, G.J. (2000) Removing allometric effects of body size in morphological analysis. *Journal of Theoretical Biology*, 205, 85–93.
https://doi.org/10.1006/jtbi.2000.2043
Mahony, M.J. (1993) The status of frogs in the Watagan Mountains area, the Central Coast of New South Wales. *In*: Lunney, D. & Ayers, D. (Eds.), *Herpetology in Australia: a diverse discipline.* Royal Zoological Society of New South Wales, Mosman, New South Wales, pp. 257–264.
https://doi.org/10.7882/RZSNSW.1993.039
Mahony, M.J., Hamer, A.J., Pickett, E.J., McKenzie, D.J., Stockwell, M.P., Garnham, J.I., Keely, C.C., Deboo, M.L., O'Meara, J. & Pollard, C.J. (2013) Identifying conservation and research priorities in the face of uncertainty: a review of the threatened bell frog complex in eastern Australia. *Herpetological Conservation and Biology*, 8, 519–538.
Martin, A. & Littlejohn, M.J. (1966) The breeding biology and larval development of *Hyla jervisiensis* (Anura: Hylidae). *Proceedings of the Linnean Society of New South Wales*, 91, 47–57.
Miller, M.A., Pfeiffer, W. & Schwartz, T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *In*: *Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, Louisiana*, 14 November 2010, pp. 1–8.
Nguyen, L.-T., Schmidt, H.A., von Haeseler, A. & Minh, B.Q. (2015) IQ-TREE: A fast and effective stochastic algorithm for estimating maximum likelihood phylogenies. *Molecular Biology Evolution*, 32, 268–274.
https://doi.org/10.1093/molbev/msu300
Penman, T., Lemckert, F. & Mahony, M. (2004) Two hundred and ten years of looking for giant burrowing frog. *Australian Zoologist*, 32, 597–604.
Penman, T., Lemckert, F. Slade, D. & Mahony, M. (2005) Non-breeding habitat requirements of the giant burrowing frog, *He-*

leioporus australiacus (Anura: Myobatrachidae) in south-eastern Australia. *Australian Zoologist*, 33, 151–157. https://doi.org/10.1109/GCE.2010.5676129
Penman, T.D. (2005) Applied conservation biology of a threatened forest dependent frog, *Heleioporus australiacus*. Thesis, The University of Newcastle, New Castle, New South Wales, 264 pp. [please cite the total page number]
Pritchard, J.K., Stephens, M. & Donnelly, P. (2000) Inference of population structure using multilocus genotype data. *Genetics*, 155, 945–959.
Puschendorf, R., Hodgson, L., Alford, R.A., Skerratt, L.F., VanDerWal, J. & Green, D. (2013) Underestimated ranges and overlooked refuges from amphibian chytridiomycosis. *Diversity and Distributions*, 19, 1313–1321. https://doi.org/10.1111/ddi.12091
Raman, H., Raman, R., Kilian, A., Detering, F., Carling, J., Coombes, N., Diffey, S., Kadkol, G., Edwards, D., McCully, M., Ruperao, P., Parkin, I.A.P., Batley, J., Luckett, D.J. & Wratten, N. (2014) Genome-wide delineation of natural variation for pod shatter resistance in *Brassica napus*. *PLoS ONE*, 9, e101673. https://doi.org/10.1371/journal.pone.0101673.
Rambaut, A., Drummond, A.J., Xie, D., Baele, G. & Suchard, M.A. (2018) Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology,* 67, 901–904. https://doi.org/10.1093/sysbio/syy032
RavenPro (2011) RavenPro: Interactive Sound Analysis Software. Version 1.4. Computer software. Center for Conservation Bioacoustics. The Cornell Lab of Ornithology. Ithaca, New York. Available from: http://ravensoundsoftware.com/ (accessed 8 September 2020)
Renner, S.S. (2016) Return to Linnaeus's focus on diagnosis, not description: the use of DNA characters in the formal naming of species. *Systematic Biology*, 65, 1085–1095. https://doi.org/10.1093/sysbio/syw032
Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A. & Huelsenbeck, J.P. (2012) MRBAYES 3.2: Efficient Bayesian phylogenetic inference and model selection across a large model space. *Systematic Biology*, 61, 539–542. https://doi.org/10.1093/sysbio/sys029
Scheele, B.C., Guarino, F., Osborne, W., Hunter, D.A., Skerratt, L.F. & Driscoll, D.A. (2014a) Decline and re-expansion of an amphibian with high prevalence of chytrid fungus. *Biological Conservation*, 170, 86–91. https://doi.org/10.1016/j.biocon.2013.12.034
Scheele, B.C., Hunter, D.A., Grogan, L. F., Berger, L., Kolby, J.E., McFadden, M.S., Marantelli, G., Skerratt, L.F. & Driscoll, D.A. (2014b) Interventions for reducing extinction risk in chytridiomycosis-threatened amphibians. *Conservation Biology*, 28, 1195–1205. https://doi.org/10.1111/cobi.12322
Skerratt, L.F., Berger, L., Speare, R., Cashins, S., McDonald, K.R., Phillott, A.D., Hines, H.B. & Kenyon, N. (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth*, 4, 125. https://doi.org/10.1007/s10393-007-0093-5
Stamatakis, A. (2014) RAxML Version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, 30, 1312–1313. https://doi.org/10.1093/bioinformatics/btu033
Stewart, D. (2000) *Australian Frog Calls. Subtropical East*. Available from: https://www.discogs.com/David-Stewart-Australian-Frog-Calls-Subtropical-East/release/6793746 (accessed 8 September 2020)
Swofford, D.L. (2003) *PAUP•. Phylogenetic analysis* using *parsimony (•and other methods). Version 4.* Sinauer Associates, Sunderland, Massachusetts. [program]
Thorpe, R.S. (1976) Biometric analysis of geographic variation and racial affinities. *Biological Reviews*, 51, 407–452. https://doi.org/10.1111/j.1469-185X.1976.tb01063.x
Trifinopoulos, J., Nguyen, L.-T., von Haeseler, A. & Minh, B.Q. (2016) W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Research*, 44 (W1), W232–W235. https://doi.org/10.1093/nar/gkw256
Tyler, M.J. & Davies, M. (1978) Species-groups within the Australopapuan hylid frog genus *Litoria* Tschudi. *Australian Journal of Zoology*, Supplementary Series, No. 63**,** 1–47.
UNESCO (2020) Blue Mountains World Heritage Area. Available from: http://whc.unesco.org/en/list/ (accessed 8 September 2020)
Watters, J.L., Cummings, S.T., Flanagan, R.L. & Siler, C.D. (2016) Review of morphometric measurements used in anuran species descriptions and recommendations for a standardized approach. *Zootaxa*, 4072 (4), 477–495. https://doi.org/10.11646/zootaxa.4072.4.6
White, A.M., Whitford, R.W. & Watson, G.F. (1980) Re-description of the Jervis Bay Tree Frog *Litoria jervisiensis* (Anura: Hylidae), with notes on the identity of Krefft's Frog (*Litoria krefftii*). *The Australian Zoologist*, 20, 375–390.
White, A.M., Whitford, R.W. & Mahony, M.J. (1994) A new species of *Litoria* (Anura: Hylidae) from eastern Australia. *Proceedings of the Linnean Society of New South Wales*, 114, 3–10.