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A new species of barred frog, *Mixophyes* (Anura: Myobatrachidae) from south-eastern Australia identified by molecular genetic analyses

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Abstract

Mixophyes are large ground-dwelling myobatrachid frogs from eastern Australia and New Guinea. Several of the species found in mid-eastern and south-eastern Australia are listed as threatened, due largely to declines presumably caused by the amphibian disease chytridiomycosis. Given the wide distribution of several of these species and that their distributions cross well-known biogeographic boundaries that often correspond to deep genetic breaks or species boundaries among closely related vertebrates, we undertook a molecular genetic assessment of population structure across the range of each species to determine the presence of undescribed species. Of the four species of *Mixophyes* subject to molecular population genetic analyses, one, the Stuttering Frog (*Mixophyes balbus*), showed a level of diversity consistent with the presence of two species. Morphometric, meristic and bioacoustic analyses corroborate these distinctions, and a new species is described for the populations south of the Macleay River valley in mid-eastern New South Wales to east Gippsland in Victoria. Applying the IUCN Red List threat criteria the new species meets the conservation status assessment criteria for Endangered 2B1a,b because its extent of occupancy and area of occupancy are below the threshold value and it has declined and disappeared from the southern two thirds of its distribution over the past 30 years.

Key words: IUCN threat category; morphology; mtDNA, nuclear DNA sequences; species boundaries

Introduction

Members of the Australo-Papuan myobatrachid genus *Mixophyes* Günther, 1864 are large ground-dwelling frogs, commonly known as "barred river frogs" or "barred frogs" due to the distinctive dark cross-bars on their arms and legs, and their association with flowing streams (Donnellan *et al.* 1990, Cogger 2014). At present, *Mixophyes* comprises seven species from eastern Australia, *M. balbus* Straughan, *M. carbinensis* Mahony, Donnellan, Richards, & McDonald, *M. coggeri* Mahony, Donnellan, Richards, & McDonald, *M. fasciolatus* Günther, *M. fleayi* Corben & Ingram, *M. iteratus* Straughan, and *M. schevilli* Loveridge, and a single southern New Guinean species *M. hihihorlo* Donnellan, Mahony & Davies.

Several species of *Mixophyes* occur across well-known biogeographic boundaries in eastern Australia and are therefore candidates to be cryptic species complexes or may show deep phylogeographic divergence as has been demonstrated in other Australian amphibians with similar distributions (Donnellan *et al.* 1999, Mahony *et al.* 2001, Donnellan & Mahony 2004, Knowles *et al.* 2004, Mahony *et al.* 2020, Mahony *et al.* 2021). Furthermore, in the Australian Wet Tropics of north-eastern Queensland where a well-documented cryptic biogeographic boundary influences population structure in most wet forest vertebrates (reviewed by Moritz *et al.* 2009), we demonstrated

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that the formerly single taxon *M. schevilli* comprises three partially co-occurring species (Mahony *et al.* 2006). None of the southern Australian species, which include three threatened taxa, have been the subject of genetic analysis of population structure.

Our aim was to use a combination of mitochondrial and nuclear molecular genetic markers to assess population structure and history in *Mixophyes* from mid-eastern Queensland south to the southern margin of the range of the genus. We focused on the three species presently considered either Endangered, *M. fleayi* and *M. iteratus*, or Vulnerable *M. balbus* (IUCN 2004, 2009. Gillespie *et al.* 2020, Geyle *et al.* 2021), but also included the more commonly encountered *M. fasciolatus* which is not considered to be threatened but has some natural disjunctions in its distribution. Our approach was to make an initial assessment of range wide population structure with mtDNA and then, if deep phylogeographic structure was observed to test the systematic status of these with nuclear single nucleotide polymorphism (SNP) data. Subsequently we only observed deep phylogenetic structure within *M. balbus* which on further genetic analysis and evaluation of morphological and acoustic variation led us to conclude that *M. balbus* comprises two species, one of which we describe as new herein.

Material and methods

For the convenience of the reader, below we use the final specific epithets in the illustrations and tables rather than use an initial group nomenclature, that we would then change to the final specific epithets in the taxonomy section. We did not assume the separate species status of groups, but rather used the results section to test this hypothesis before presenting the final taxonomy.

Specimens for molecular genetic analyses were collected from across the range of each of four species of *Mixophyes* (Table 1). Samples from the southern portion of the historic range of *M. balbus* were not available as no extant populations are presently known in Victoria and southern New South Wales (NSW). Tissue for molecular genetic analysis included liver, heart, tadpole tail tip or a 2 mm diameter biopsy taken from the webbing between the second and third toe in adults using a sterile tissue biopsy punch (Kai dermal biopsy punch, sterile disposable, 2 mm).

mtDNA analysis. DNA was extracted with a Gentra Purgene Kit (Qiagen). The *tRNA*^{GLN} and *tRNA*^{MET} genes and part of the *ND2* gene were PCR amplified and directly sequenced with the primers forward tRNA^{ILE} 5'-AAG GAC CTC CTT GAT AGG GA-3' and reverse ND2 5'-ATTTTTCGTAGTTGGGTTTGRTT-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.

For model-based phylogenetic inference, we estimated the best substitution model and partition scheme from three data subsets of the 1st, 2nd and 3rd codon positions, with ModelFinder (Kalyaanamoorthy *et al.* 2017) following the Bayes Information Criterion (BIC) criterion. For the maximum likelihood approach, we used IQ-tree (Nguyen *et al.* 2015) on the IQ-TREE webserver (Trifinopoulos *et al.* 2016). We assessed branch support with 100 standard bootstrap pseudo-replicates (Hoang *et al.* 2018). Bayesian analysis was conducted using MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). The analysis was run with model parameters unlinked, using default priors for ten 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 v1.4.1 (Rambaut *et al.* 2018). The first 25% of sampled trees were discarded as burn-in.

Net average sequence divergence between lineages (*dA*) 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 means.

SNP data generation. 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). DNA samples were processed in restriction enzyme digestion/ligation reactions using a combination of the *Pst*I/*Sph*I restriction enzymes and ligated fragments were PCR amplified as described by Kilian *et al.* (2012) and Mahony *et al.* (2020) for 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 3, 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. SNPs were identified within each cluster by examining parameters calculated for each sequence across all samples-primarily average and variance of sequencing depth, the average counts for each SNP allele and the call rate (proportion of samples for which the marker is scored). Where three sequences survived filtering to this point, the two variants with the highest read depth were selected (see Georges *et al.* [2018] for a more detailed description of the SNP identification process). 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 16.9 reads per individual per locus for reference alleles and 10.8 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- dMix17-2654-1) and the genotype matrix from 10.6084/m9.figshare.22067060

The SNP data and associated metadata were read into a genlight object (Jombart 2008) to facilitate processing with package dartR (Gruber *et al.* 2018). Only loci with 100% repeatability (repAvg) were chosen for subsequent analysis. Further filtering was undertaken on the basis of having a call rate <90% (unless otherwise specified) and the locus being present in at least 70% of individuals. We retained 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≤15), we did not filter loci for departures from Hardy-Weinberg equilibrium or linkage disequilibrium.

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 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, which has been shown to perform well with codominant markers such as SNPs. We used the uncorrelated allele frequency and the admixture ancestry models to assess values of *K* from 1 to 5. We performed three 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 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.

We inferred phylogenetic relationships among the samples using the concatenated SNP data set with two

TABLE 1. Collection details for all specimens used in the molecular genetic analyses.

ABTC—Australian biological tissue collection, South Australian Museum, specimen number; Local#—location number used in Fig. 1; Voucher—Museum specimen registration number (AMS—Australian Museum Sydney, QM—Queensland Museum, SAMA—South Australian Museum) and indication that voucher occurs; SF—State Forest, NP—National Park, SCA—State Conservation Area, Qld—Queensland, NSW—New South Wales, Vic—Victoria, PNG—Papua New Guinea; GenBank accession numbers (JNXXX) and y = yes; SNP—single nucleotide polymorphism y = sequenced,—not sequenced.

Taxon	ABTC	State	local#	Location	Latitude	Longitude	Voucher	GenBank	SNP
carbinensis	25116	Qld	-	Mt Lewis	-16.525	145.275	No voucher	JN677486	-
carbinensis	51064	Qld	-	Mt Lewis	-16.525	145.275	SAMA R61580	JN677489	-
carbinensis	31869	Qld	-	Windsor Tableland	-16.260	145.040	QMJ54849	JN677495	-
carbinensis	25121	Qld	-	Windsor Tableland	-16.258	145.042	SAMA R64485	JN677515	-
coggeri	104469	Qld	-	Julatten	-16.563	145.354	No voucher	OQ447341	-
coggeri	104470	Qld	-	Julatten	-16.563	145.354	No voucher	OQ447342	-
coggeri	104471	Qld	-	Julatten	-16.563	145.354	No voucher	OQ447343	-
coggeri	17497	Qld	-	Paluma	-19	146.2	SAMA R42234	OQ447340	-
coggeri	25132	Qld	-	Kirrama Range	-18.210	145.760	No voucher	JN677513	-
coggeri	51086	Qld	-	Mt Lewis	-16.570	145.250	No voucher	JN677522	-
fasciolatus	11582	Qld	-	Broken River Road, Eungella	-20.186	148.524	AMS R.126097	OQ447344	-
fasciolatus	51276	Qld	-	Pelion SF, Clarke Range	-21.100	148.750	No voucher	JN93945	-
fasciolatus	51291	Qld	-	Kroombit Tops	-24.366	151.968	SAMA R51190	OQ447359	-
fasciolatus	16977	Qld	-	Brisbane SF	-26.5	152.13	SAMA R51178	-	y
fasciolatus	26080	Qld	-	Linda Garrett Reserve, Blackall Range	-26.619	152.851	No voucher	OQ447357	-
fasciolatus	31870	Qld	-	Mt Glorious NP	-28.050	152.400	QM J56097	-	y
fasciolatus	31874	Qld	-	Mt Glorious NP	-28.050	152.400	No voucher	OQ447358	-
fasciolatus	24981	Qld	-	Cunninghams Gap	-28.056	152.378	No voucher	OQ447348	-
fasciolatus	24880	NSW	-	Caldera Road, Wollumbin SF	-28.379	153.242	No voucher	OQ447345	-
fasciolatus	24930	NSW	-	Sheepstation Creek, Border Ranges NP	-28.412	153.021	No voucher	OQ447347	-
fasciolatus	24978	NSW	-	Tooloom Scrub, Beaury SF	-28.472	152.399	No voucher	OQ447360	-
fasciolatus	25409	NSW	-	Terania Creek, Nightcap Range	-28.571	153.310	SAMA R66213	OQ447353	-
fasciolatus	25786	NSW	-	Bundoozle Road, Richmond Range	-28.609	152.713	SAMA R59909	OQ447355	-
fasciolatus	24996	NSW	-	Yabbra SF	-28.716	152.430	No voucher	OQ447349	-
fasciolatus	25639	NSW	-	Desert Creek, Washpool NP	-29.269	152.430	No voucher	OQ447354	-
fasciolatus	25088	NSW		Liberation Trail, Guy Fawkes NP	-29.883	152.317	AMS R.139334	OQ447352	-

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TABLE 1. (Continued)

Taxon	ABTC	State	local#	Location	Latitude	Longitude	Voucher	GenBank	SNP
fasciolatus	25068	NSW	-	Chaelundi Road, Marengo SF	-30.133	152.417	No voucher	suppl	-
fasciolatus	25074	NSW	-	Wild Cattle Creek SF	-30.167	152.767	No voucher	-	y
fasciolatus	25075	NSW	-	Wild Cattle Creek SF	-30.167	152.767	No voucher	OQ447351	-
fasciolatus	25824	NSW	-	Ulong Creek, Ulong	-30.258	152.898	No voucher	OQ447356	-
fasciolatus	25016	NSW	-	Carrai Plateau	-30.84	152.238	No voucher	OQ447350	-
fasciolatus	24192	NSW	-	Watagan SF	-33.12	151.17	SAMA R33920	-	y
fasciolatus	24193	NSW	-	Watagan SF	-33.12	151.17	SAMA R33921	-	y
fasciolatus	24916	NSW	-	Stoney Point Road, Watagan SF	-33.135	151.350	No Voucher	OQ447346	-
hihihorlo	45864	PNG	-	Namosado, Southern Highlands Province	-6.250	142.780	AMS R.120835	OQ447366	-
hihihorlo	45867	PNG	-	Namosado, Southern Highlands Province	-6.250	142.780	AMS R.120834	OQ447367	-
iteratus	26111	Qld	-	Linda Garrett Reserve, Blackall Range	-26.619	152.851	No Voucher	OQ447376	-
iteratus	26118	Qld	-	Linda Garrett Reserve, Blackall Range	-26.619	152.851	No Voucher	OQ447377	-
iteratus	25706	NSW	-	Byrill Creek, Mebbin NP	-28.447	153.192	SAMA R59929	OQ447372	-
iteratus	25776	NSW	-	Byrill Creek, Mebbin SF	-28.447	153.192	SAMA R59923	suppl	-
iteratus	25765	NSW	-	Peacock Creek, Richmond Range SF	-28.659	152.716	No Voucher	OQ447373	-
iteratus	17191	NSW	-	Washpool Creek, Washpool NP	-29.269	152.430	SAMA R51193	OQ447370	-
iteratus	25100	NSW	-	Boundary Creek Road, Boundary Creek SF	-29.973	152.598	AMS R.139353	OQ447371	-
iteratus	25827	NSW	-	Bruxner Park Flora Reserve	-30.241	153.095	No Voucher	OQ447374	-
iteratus	25828	NSW	-	Bruxner Park Flora Reserve	-30.241	153.095	No Voucher	OQ447375	-
iteratus	4004	NSW	-	Bellinger River, Thora	-30.450	152.620	SAMA R33676	OQ447368	-
iteratus	7142	NSW	-	Congewai Creek, Watagan NP	-32.998	151.406	AMS R.188979	suppl	-
iteratus	7123	NSW	-	The Basin, Olney SF	-33.104	151.231	SAMA R19811	OQ447369	-
fleayi	110104	Qld	f1	Bundaroo Creek, Conondale NP	-26.690	152.610	QM J86613	-	y
fleayi	127513	Qld	f2	Gap Creek West, Main Range NP	-28.049	152.383	No voucher	-	y
fleayi	127514	Qld	f2	Gap Creek West, Main Range NP	-28.049	152.383	No voucher	-	y
fleayi	25851	Qld	f3	Cunningham's Gap	-28.056	152.378	SAMA R59934	suppl	y
fleayi	26089	Qld	f3	Cunningham's Gap	-28.056	152.378	No voucher	-	y
fleayi	26090	Qld	f3	Cunningham's Gap	-28.056	152.378	No voucher	-	y
fleayi	26327	Qld	f4	Lamington NP	-28.198	153.183	AMS R.188960	OQ447361	y
fleayi	127678	Qld	f4	Lamington NP	-28.215	153.126	AMS R.188963	-	y

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TABLE 1. (Continued)

Taxon	ABTC	State	local#	Location	Latitude	Longitude	Voucher	GenBank	SNP
fleayi	139774	Qld	f5	Coomera River Circuit, Lamington NP	-28.236	153.193	No voucher	-	y
fleayi	66414	NSW	f6	Brindle Creek, Border Ranges NP	-28.380	153.030	AMR.188961	-	y
fleayi	14078	NSW	f7	Tooloom Scrub, Beaury SF	-28.480	152.420	No Voucher	OQ447362	-
fleayi	25405	NSW	f8	Terania Creek Nightcap NP	-28.571	153.314	AMS R.188962	OQ447363	y
fleayi	25853	NSW	f8	Terania Creek Nightcap NP	-28.600	153.300	SAMA R59930	-	y
fleayi	26464	NSW	f9	Little Haystack Creek, Yabbra SF	-28.710	152.510	AMS R.188964	OQ447364	y
fleayi	26465	NSW	f10	Yabbra Creek	-28.716	152.430	AMS R.188966	OQ447365	y
balbus	25041	NSW	1	Duncans Creek, Malara SF	-29.135	152.315	AMS R.188982	OQ447312	-
balbus	25042	NSW	1	Duncans Creek, Malara SF	-29.135	152.315	AMS R.188991	OQ447313	-
balbus	26455	NSW	2	Forestland SF	-29.201	152.122	AMS R.188941	OQ447314	y
balbus	26456	NSW	2	Forestland SF	-29.201	152.122	AMS R.188990	suppl	-
balbus	26458	NSW	2	Forestland SF	-29.201	152.122	AMS R.188940	OQ447315	-
balbus	26459	NSW	2	Forestland SF	-29.201	152.122	AMS R. 188984	OQ447316	-
balbus	26460	NSW	3	Rockadooie Creek, Butterleaf SCA	-29.433	152.138	AMS R.188986	OQ447320	y
balbus	26278	NSW	3	Rockadooie Creek, Butterleaf SCA	-29.433	152.138	AMS R.188942	OQ447317	-
balbus	26279	NSW	3	Rockadooie Creek, Butterleaf SCA	-29.433	152.138	AMS R.188943	OQ447318	-
balbus	26280	NSW	3	Rockadooie Creek, Butterleaf SCA	-29.433	152.138	AMS R.188992	-	-
balbus	26461	NSW	3	Rockadooie Creek, Butterleaf SCA	-29.433	152.138	AMS R.188939	-	-
balbus	26462	NSW	3	Rockadooie Creek, Butterleaf SCA	-29.433	152.138	AMS R.188993	-	-
balbus	26463	NSW	3	Rockadooie Creek, Butterleaf SCA	-29.433	152.138	AMS R.188983	-	-
balbus	25053	NSW	4	Curramore SF	-29.467	152.167	AMS R.139368	OQ447324	-
balbus	26454	NSW	5	Seven Mile Creek, Curramore SF	-29.467	152.067	AMS R.188985	-	y
balbus	25644	NSW	6	Coombadjha Creek, Washpool NP	-29.472	152.322	No Voucher	OQ447326	y
balbus	25645	NSW	6	Coombadjha Creek, Washpool NP	-29.472	152.322	No Voucher	OQ447327	-
balbus	25646	NSW	6	Coombadjha Creek, Washpool NP	-29.472	152.322	No Voucher	OQ447328	-
balbus	25081	NSW	7	Liberation Trail, Guy Fawkes NP	-29.883	152.317	AMS R.139327	OQ447329	y
balbus	25082	NSW	7	Liberation Trail, Guy Fawkes NP	-29.883	152.317	AMS R.139328	OQ447330	-
balbus	25083	NSW	7	Liberation Trail, Guy Fawkes NP	-29.883	152.317	AMS R.139329	OQ447331	-
balbus	25089	NSW	7	Liberation Trail, Guy Fawkes NP	-29.883	152.317	AMS R.139335	OQ447332	-
balbus	25090	NSW	7	Liberation Trail, Guy Fawkes NP	-29.883	152.317	AMS R.139336	OQ447333	-

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TABLE 1. (Continued)

Taxon	ABTC	State	local#	Location	Latitude	Longitude	Voucher	GenBank	SNP
balbus	25091	NSW	7	Liberation Trail, Guy Fawkes NP	-29.883	152.317	AMS R.139337	OQ447334	-
balbus	25092	NSW	7	Liberation Trail, Guy Fawkes NP	-29.883	152.317	AMS R.139338	OQ447335	-
balbus	25069	NSW	8	Marengo SF	-30.133	152.417	No Voucher	OQ447336	y
balbus	25073	NSW	9	Wild Cattle Creek SF	-30.167	152.767	No Voucher	-	y
balbus	25076	NSW	9	Wild Cattle Creek SF	-30.167	152.767	No Voucher	OQ447337	y
balbus	25572	NSW	9	Wild Cattle Creek SF	-30.167	152.767	No Voucher	OQ447338	-
balbus	90581	NSW	10	2.1km N Shepherds Falls, near Dorrigo	-30.383	152.732	SAMA R66178	OQ447339	y
balbus	25070	NSW	11	Styx River SF	-30.550	152.333	No Voucher	suppl	y
balbus	104274	NSW	11	Styx River catchment	-30.550	152.333	AMS R.188760	-	y
balbus	104275	NSW	11	Styx River catchment	-30.550	152.333	AMS R.188761	-	y
australis	25039	NSW	12	near Kookaburra Carrai Plateau	-31.020	152.336	No Voucher	OQ447378	y
australis	25040	NSW	12	near Kookaburra Carrai Plateau	-31.020	152.336	No Voucher	OQ447379	
australis	125996	NSW	13	Birds Nest Creek, Oxley Wild Rivers	-31.048	152.168	No voucher	-	y
australis	125997	NSW	13	Birds Nest Creek, Oxley Wild Rivers	-31.048	152.168	No voucher	-	y
australis	24978	NSW	14	Mt Boss SF	-31.183	152.367	No Voucher	OQ447360	-
australis	24966	NSW	14	Mt Boss SF	-31.183	152.367	No Voucher	-	y
australis	25049	NSW	14	Mt Boss SF	-31.183	152.367	No Voucher	-	y
australis	25831	NSW	15	Dingo SF	-31.707	152.131	SAMA R59916	OQ447381	y
australis	25472	NSW	16	Devils Hole, Barrington Tops	-31.910	151.480	SAMA R59925	OQ447382	y
australis	25473	NSW	16	Devils Hole, Barrington Tops	-31.916	151.482	SAMA R59921	OQ447383	y
australis	141372	NSW	17	Sharpes Creek, Barrington Range	-32.040	151.640	AMS R.188748	-	y
australis	141373	NSW	17	Sharpes Creek, Barrington Range	-32.040	151.640	AMS R.188749	-	y
australis	141375	NSW	17	Sharpes Creek, Barrington Range	-32.040	151.640	AMS R.188751	-	y
australis	25321	NSW	18	Sharpes Creek, Gloucester Tops	-32.057	151.679	SAMA R66216	-	y
australis	25322	NSW	18	Sharpes Creek, Gloucester Tops	-32.057	151.679	SAMA R66217	-	y
australis	25323	NSW	18	Sharpes Creek, Gloucester Tops	-32.057	151.679	SAMA R66218	-	y
australis	141446	NSW	19	Wards River, The Glen Nature Reserve	-32.166	152.050	AMS R.189000	-	y
australis	141447	NSW	19	Wards River, The Glen Nature Reserve	-32.166	152.050	AMS R.189000	-	y
australis	141449	NSW	19	Wards River, The Glen Nature Reserve	-32.166	152.050	AMS R.189000	-	y

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TABLE 1. (Continued)

Taxon	ABTC	State	local#	Location	Latitude	Longitude	Voucher	GenBank	SNP
australis	141450	NSW	19	Wards River, The Glen Nature Reserve	-32.166	152.050	AMS R.189000	-	y
australis	141451	NSW	19	Wards River, The Glen Nature Reserve	-32.166	152.050	AMS R.189000	-	y
australis	141452	NSW	19	Wards River, The Glen Nature Reserve	-32.167	152.032	AMS R.189000	-	y
australis	141453	NSW	19	Wards River, The Glen Nature Reserve	-32.167	152.032	AMS R.189000	-	y
australis	141454	NSW	19	Wards River, The Glen Nature Reserve	-32.167	152.032	AMS R.189000	-	y
australis	141455	NSW	19	Wards River, The Glen Nature Reserve	-32.167	152.032	AMS R.189000	-	y
australis	141456	NSW	19	Wards River, The Glen Nature Reserve	-32.167	152.032	AMS R.189000	-	y
australis	141457	NSW	19	Wards River, The Glen Nature Reserve	-32.167	152.032	AMS R.189000	-	y
australis	141439	NSW	20	Waukivory Creek, The Glen Nature Reserve	-32.173	152.079	AMS R.189179	-	y
australis	141440	NSW	20	Waukivory Creek, The Glen Nature Reserve	-32.173	152.079	AMS R.189179	-	y
australis	141441	NSW	20	Waukivory Creek, The Glen Nature Reserve	-32.173	152.079	AMS R.188999	-	y
australis	141442	NSW	20	Waukivory Creek, The Glen Nature Reserve	-32.173	152.079	AMS R.188999	-	y
australis	141443	NSW	20	Waukivory Creek, The Glen Nature Reserve	-32.173	152.079	AMS R.188999	-	y
australis	141444	NSW	20	Waukivory Creek, The Glen Nature Reserve	-32.173	152.079	AMS R.188999	-	y
australis	141445	NSW	20	Waukivory Creek, The Glen Nature Reserve	-32.173	152.079	AMS R.188999	-	y
australis	141383	NSW	21	Mt Royal, Upper Glendon Brook	-32.204	151.329	AMS R.188995	-	y
australis	141384	NSW	21	Mt Royal, Upper Glendon Brook	-32.204	151.329	AMS R.188995	-	y
australis	141385	NSW	21	Mt Royal, Upper Glendon Brook	-32.204	151.329	AMS R.188995	-	y
australis	141386	NSW	21	Mt Royal, Upper Glendon Brook	-32.204	151.329	AMS R.188995	-	y
australis	141387	NSW	21	Mt Royal, Upper Glendon Brook	-32.204	151.329	AMS R.188995	-	y
australis	141388	NSW	21	Mt Royal, Upper Glendon Brook	-32.204	151.329	AMS R.188995	-	y
australis	141389	NSW	21	Mt Royal, Upper Glendon Brook	-32.204	151.329	AMS R.188995	-	y
australis	141390	NSW	21	Mt Royal, Upper Glendon Brook	-32.204	151.329	AMS R.188995	-	y
australis	141391	NSW	22	Mt Royal, Fal Brook	-32.226	151.272	AMS R.188998	-	y
australis	141392	NSW	22	Mt Royal, Fal Brook	-32.226	151.272	AMS R.188998	-	y
australis	141393	NSW	22	Mt Royal, Fal Brook	-32.226	151.272	AMS R.188998	-	y
australis	141394	NSW	22	Mt Royal, Fal Brook	-32.226	151.272	AMS R.188998	-	y
australis	141395	NSW	22	Mt Royal, Fal Brook	-32.226	151.272	AMS R.188998	-	y
australis	141376	NSW	23	Frying Pan Creek, Chichester SF	-32.230	151.760	AMS R.188938	-	y
australis	141377	NSW	23	Frying Pan Creek, Chichester SF	-32.230	151.760	No voucher	-	y

.....continued on the next page

TABLE 1. (Continued)

Taxon	ABTC	State	local#	Location	Latitude	Longitude	Voucher	GenBank	SNP
australis	141378	NSW	23	Frying Pan Creek, Chichester SF	-32.230	151.760	No voucher	-	y
australis	141379	NSW	23	Frying Pan Creek, Chichester SF	-32.230	151.760	No voucher	-	y
australis	104300	NSW	24	Myall River SF	-32.370	152.101	AMS R.189049	OQ447387	y
australis	104301	NSW	24	Myall River SF	-32.370	152.101	AMS R.189050	suppl	y
australis	104302	NSW	24	Myall River SF	-32.370	152.101	AMS R.189047	OQ447388	y
australis	104303	NSW	24	Myall River SF	-32.370	152.101	AMS R.189048	OQ447389	y
australis	7124	NSW	25	Watagan State Forest	-33.030	151.330	SAMA R19812	OQ447390	y
australis	80545	NSW	26	Ruby Creek, Mt Werong	-34.083	149.946	No voucher	-	y
australis	80546	NSW	26	Ruby Creek, Mt Werong	-34.083	149.946	No voucher	suppl	y
australis	80547	NSW	26	Ruby Creek, Mt Werong	-34.083	149.946	No voucher	OQ447391	y
australis	7307	NSW	27	Macquarie Rivulet, Macquarie Pass	-34.560	150.650	No voucher	OQ447392	y
australis	7309	NSW	27	Macquarie Rivulet, Macquarie Pass	-34.560	150.650	No voucher	OQ447393	y
australis	101748	NSW	27	Macquarie Rivulet, Macquarie Pass	-34.560	150.650	No voucher	OQ447394	-
australis	101749	NSW	27	Macquarie Rivulet, Macquarie Pass	-34.560	150.650	No voucher	OQ447395	-
australis	101751	NSW	27	Macquarie Rivulet, Macquarie Pass	-34.560	150.650	No voucher	OQ447396	-
australis	101752	NSW	27	Macquarie Rivulet, Macquarie Pass	-34.560	150.650	No voucher	OQ447397	-
australis	101753	NSW	27	Macquarie Rivulet, Macquarie Pass	-34.560	150.650	No voucher	OQ447398	-
australis	101754	NSW	27	Macquarie Rivulet, Macquarie Pass	-34.560	150.650	No voucher	OQ447399	-
schevilli	25112	Qld	-	Lamb Range	-17.11	145.63	No voucher	JN677494	
schevilli	25114	Qld	-	Lamb Range	-17.11	145.63	No voucher	JN677491	
schevilli	25125	Qld	-	Massey Creek	-17.61	145.56	No voucher	JN677523	
schevilli	25130	Qld	-	Lake Eacham	-17.28	145.63	No voucher	OQ447400	
schevilli	25136	Qld	-	Big Tableland	-15.7	145.27	No voucher	JN677501	
schevilli	31884	Qld	-	Thornton Peak	-16.2	145.33	QM J56102	JN677519	
Limnodynastes salmini	-		-	-	-	-	-	JX564877	-
Neobatrachus pictus	16199	Vic	-	Wartook	-37.03	142.35	S_R43073	OQ447401	-
Lechriodus melanopyga	-	PNG	-	-	-	-	MCN_DNA_8001	JF703230	-

phylogenetic tree building methods suited to SNP data, SVDquartets and maximum likelihood. For the SVDquartets analysis we performed three independent runs with sampling of all possible quartets with the program PAUP* version 4.0a build 165 (Swofford 2003) to assess topological convergence, in which run included 100 bootstrap replicates.

For the maximum likelihood approach, we used IQ-tree (Nguyen *et al.* 2015), 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 (Leach *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.* 2018).

Morphology. Specimens examined are deposited in the Australian Museum, Sydney (AMS); Australian National Wildlife Collection, CSIRO Lyneham (ANWC); Museum of Comparative Zoology, Harvard University (MCZ); Museums Victoria, Melbourne (MV); Queensland Museum, Brisbane (QM); and the South Australian Museum, Adelaide (SAMA).

Morphometric measurements of 183 preserved adult specimens (139 males, 44 females) were taken with callipers to the nearest mm for the following 12 traits following Donnellan *et al.* (2021): SVL—snout-to-vent length, HL—head length, HW—head width, TD—tympanum diameter, ED—eye length, EN—eye to naris distance, IOD—inter-orbital distance, IND—internarial distance, FLL—forearm length, Fin3L—third finger length, TL—tibia length, IMT–inner metatarsal tubercle length (Supplementary Table S2) (for all Supplementary material hereafter refer to Figshare. 10.6084/m9.figshare.22067060). Sex was determined by visual inspection of gonads or the presence of nuptial pads in males. Morphometric data are expressed as means ± one standard deviation and ranges.

For the multivariate analyses, sexes were analysed separately and 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 the mean of the regression coefficients estimated independently for each taxon from logarithmically transformed values of x_i and y_i (see Thorpe 1976, Lleonart *et al.* 2000). We used the R script *GroupStruct* with the species option to produce the adjusted mensural data (Chan & Grismer 2022).

Linear Discriminant Analysis (LDA) was undertaken to assess whether the taxa distinguished by the genetic analyses also differed in body shape. LDA was performed after log transforming all variables after they had been adjusted for size/growth as described above using the 'lda' function from v7.3-40 of the R package MASS in rStudio version 0.98.1028. We chose to use LDA as specimens could be allocated to putative taxa based on the results of the molecular genetic analysis and distribution.

Reproductive call. We made recordings during our field observations and accessed recordings from several published sources (Table 2). Recordings were made on several different recorders including analogue and digital devices (Table 2). Wherever possible, when specimens were collected for genetic and morphological comparisons, we also recorded male calls (Table 2). Where possible at least five calls per individual male were analysed and the mean values calculated (Table 2), prior to obtaining population / species means.

Recordings of *M. balbus* were obtained at the Little Styx River, which is within 5 km of the type locality near Point Lookout New England National Park (Straughan 1968). We also used the values that Straughan (1966) calculated from recordings of this species that he made at the type locality (Straughan's original recordings were not available). We obtained a recording from the Cann River Victoria, made in 1965, from the sound library of Dr Murray Littlejohn (Littlejohn 1969), since it represents the only call recording from a population at the far south of the species range.

Analogue recordings were converted to digital files using a direct line from a Sony WMD60 cassette recorded to a Marantz PMD60 recorder at a sampling rate of 32,000Hz. Sound analysis was conducted with Raven Pro v1.6. Audiospectrograms for analysis were calculated and illustrated with fast-Fourier transform (FFT) of 512 points, 50% overlap and 172 Hz grid-spacing, using Hamming windows. In describing the reproductive calls, we use a call centred approach and the definitions of acoustic features of Köhler *et al.* (2017), and the sound categories of Beeman (1998). For comparisons of call structure, i.e., note and inter-note duration and note amplitude modulation, all calls were viewed with the time axis held at 5 seconds, so that the note trains and pulse structure of notes could be directly compared. Temperature is known to affect temporal characteristic such as pulse length and pulse rate and less so structural traits such as the number of pulses or dominant frequency (Gerhardt & Huber 2002). Calls

were corrected for difference in temperature using linear regression and linear relationships were found in duration of note 1 and 2 and note repetition rate for note 1, but not note 2, in the calls of northern and southern groups. Regressions were tested for homogeneity of slope between the northern and southern group prior to using statistical tests to compare temporal call traits.

All five species of *Mixophyes* in south-eastern Australia produce an encounter call (Barker *et al.* 1995, Mahony unpubl. data), also termed aggressive or territorial calls (Wells 1977) that may be produced independently or in conjunction with the advertisement call (= female attractant component of the call). The encounter call in *Mixophyes* may be inserted between advertisement calls, and in some cases be repeated, or it may be produced independently, particularly when two males are in close proximity. Although female choice experiments were not conducted we assume that the most commonly uttered call is the advertisement call (Gerhardt *et al.* 2007). To assess the function of the encounter call in *M. balbus*, we made observations of acoustic interactions between males in the field and we also used play back of both call components to adult males in nature to determine responses to each component (Mahony unpubl. data).

Assessment of the IUCN Red List threat category. We assessed the IUCN Red List threat category for each species using the IUCN (2019) criteria (Supplementary Text). Contractions have been reported in the distribution of *M. balbus* (Mahony 1993, Hero *et al.* 2006, Hunter & Gillespie 2011). To make an updated and quantitative assessment of whether there have been changes in distribution over time we calculated the Area of Occupancy (AOO) and Extent of Occupancy (EOO: minimum convex hull and alpha hull) following IUCN (2019). AOO was calculated using a 2 sq km grid cell, since radio-tracking and mark-recapture studies show that adult males do not move more than about 100 m and remain in the riparian zone, while adult females may move several hundred meters from the stream where breeding occurs (Lemckert & Morse 1999). Furthermore, our field observations are that tadpoles are not washed downstream at times of high-water flow. The 2 sq km grid therefore probably overestimates the smallest area required for the survival of any life stage (IUCN 2019), and our estimates of AOO should be conservative.

All records of *M. balbus* in the Atlas of Living Australia (ALA—accessed June 2022) and several other databases (Supplementary Text) were mapped, and five spurious records and 13 records without adequate location information were removed from the total of 2123 unique occurrence records. Records were then divided into several time periods: up to and including 1980 (including all records prior to 1980), and for each subsequent decade up to and including 2020. These periods were chosen since it is established that the introduced pathogen amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) (Bd) 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 these periods. A decade is between the upper and lower values (8 and 12 years) considered for three generations in these species (see Supplementary Text).

Results

Mitochondrial DNA. The 610 bp alignment comprised 132 bp of the $tRNA^{GLN}$ and $tRNA^{Met}$ genes and 477bp of the *ND2* gene. A single partition with the HKY+F+G4 model was selected. In the mitochondrial phylogenetic tree (Fig. 2), sequences from *M. balbus* fell into two major clades: clade 1 includes samples from the north of the distribution, from the Girard State Forest, New England Range in the north to the Dingo Tops (Tapin Tops National Park and Doyles River State Forest) in the south, and clade 2 includes samples from the Barrington and Myall Ranges south to the Illawarra Region in NSW (Fig. 2). We did not find any locations where the members of the two clades were sympatric. The closest localities for the two clades were localities 15 (Dingo Tops) and 16 (Barrington Tops) separated by 67 km (Fig. 1). Net average sequence divergence (*dA*) between the clades was 0.02.

In contrast to *M. balbus*, three other species, *M. fleayi*, *M. fasciolatus* and *M. iteratus* each show low levels of divergence amongst their *ND2* sequences with mean within group distances of 0.005, 0.009 and 0.003 respectively. To investigate the systematic implications of the deep mtDNA sequence divergence within *M. balbus*, we obtained nuclear SNP genotypes for samples from across the species range.

SNP analyses. 92 individuals of *M. balbus*, *M. fleayi* and the outgroup *M. fasciolatus* were genotyped successfully for 62,949 loci. Firstly, we filtered to retain as many outgroup (*M. fasciolatus*) individuals as possible for phylogenetic analysis (a total of 88 individuals with 40,043 loci), with a second filtering of the ingroup (*M. balbus* and *M. fleayi*) only (a total of 77 individuals with 19,680 loci) for PCoA and STRUCTURE analyses.

TABLE 2. Advertisement call traits of *Mixophyes*. For details of call traits see Materials and Methods. *Data from the type locality of *M. balbus* recorded by Straughan (1966), and **Cann River, Victoria from the far south of the distribution recorded by Littlejohn (1987). DF—dominant frequency (Hz). N—the number of males recorded. Pulse rate (pulses per second). NP—National Park. nd—not determined. [1]recorded on a Sony Walkman WMD60 with a Sony Electret microphone, [2]recorded on a Marantz PMD60 with a Rode N1 directional microphone. [3]Recorded by D. Stewart, Sound Devices with Sennheiser MKE600 directional microphone. Note duration and note repetition rate were corrected for temperature. Call recordings from several locations for both species have been deposited at the Australian Museum as multimedia records, and the call recording for *M. australis* **sp. nov.** from the type locality is attached to the database record for R188750.

					Trait							
					Note 1				Note 2			
Location	Lat.	Long.	N	Temp °C	Duration (s), temp corrected	Number of pulses	Pulse rate, temp corrected	DF	Duration (s), temp corrected	Number of pulses	Pulse rate, not temp corrected	DF
Northern—*M. balbus* (N=23)												
[1]Duncan's Creek, Timbarra Plateau	-29.12	152.30	2	17	0.70	14.8	16.62	767	0.39	11.5	26.7	768
[2]Coombadjha Creek, Washpool NP	-29.47	152.32	4	17	0.70	10.8	16.62	798	0.38	8.2	18.1	775
[1]Rockadooie Creek, Curramore NP	-29.47	152.07	2	18	0.55	13.2	18.28	890	0.33	9.0	19.4	775
[1]Liberation Fire Trail, Forestland SF	-29.88	152.32	2	nd	0.55	13.1	18.28	753	0.33	9.2	27.8	742
[2]Little Styx River, New England NP	-30.48	152.38	2	18	0.56	12.5	18.28	747	0.33	9.1	20.7	731
*Point Lookout, New England NP	-30.50	152.37	11	12	1.42	11.0	8.32	950	0.65	9.0	15.0	nd
Mean					0.68	12.56	16.07	817.5	0.41	9.09	21.28	758
Southern—*M. australis* **sp. nov** (N=11)												
[2]Birds Nest Creek, Oxley Wild Rivers NP	-31.03	152.17	2	16	0.84	13.4	14.96	732	0.44	8.8	26.7	692
Dilgry River, Barrington NP	-31.90	151.47	1	14	1.17	8.5	11.65	774	nd	5.0	nd	nd
[1]Sharpes Creek, Gloucester Tops NP	-32.05	151.67	4	18	0.56	5.6	18.28	734	0.33	5.0	17.8	731
[2]Frying Pan Creek, Chichester SF	-32.22	151.75	2	17	0.70	7.0	16.62	703	0.39	6.0	nd	847
[1]Gap Creek, Watagan NP	-34.02	152.42	1	18	0.56	8.7	18.28	716	0.33	5.0	18.1	697
**Vic/NSW Border, 500 yards on Cann River Road	-37.23	149.27	1	12	1.43	7.0	8.33	757	0.65	5.76	17.9	740
Mean					0.	8.64	15.96	731	0.45	6.2	20.86	724
Mixophyes fleayi (N=3)												
[3]Rocky Creek, Lamington NP	-28.20	153.12	1	18	0.38	4.7	12.41	787	0.38	3.4	11.04	756
[1]Haystacks Creek, Tooloom Range	-28.65	152.48	2	18	0.61	9.0	14.75	650	0.28	7.0	25.0	628
Mean					0.5	6.8	13.6	718	0.29	5.2	18.02	692

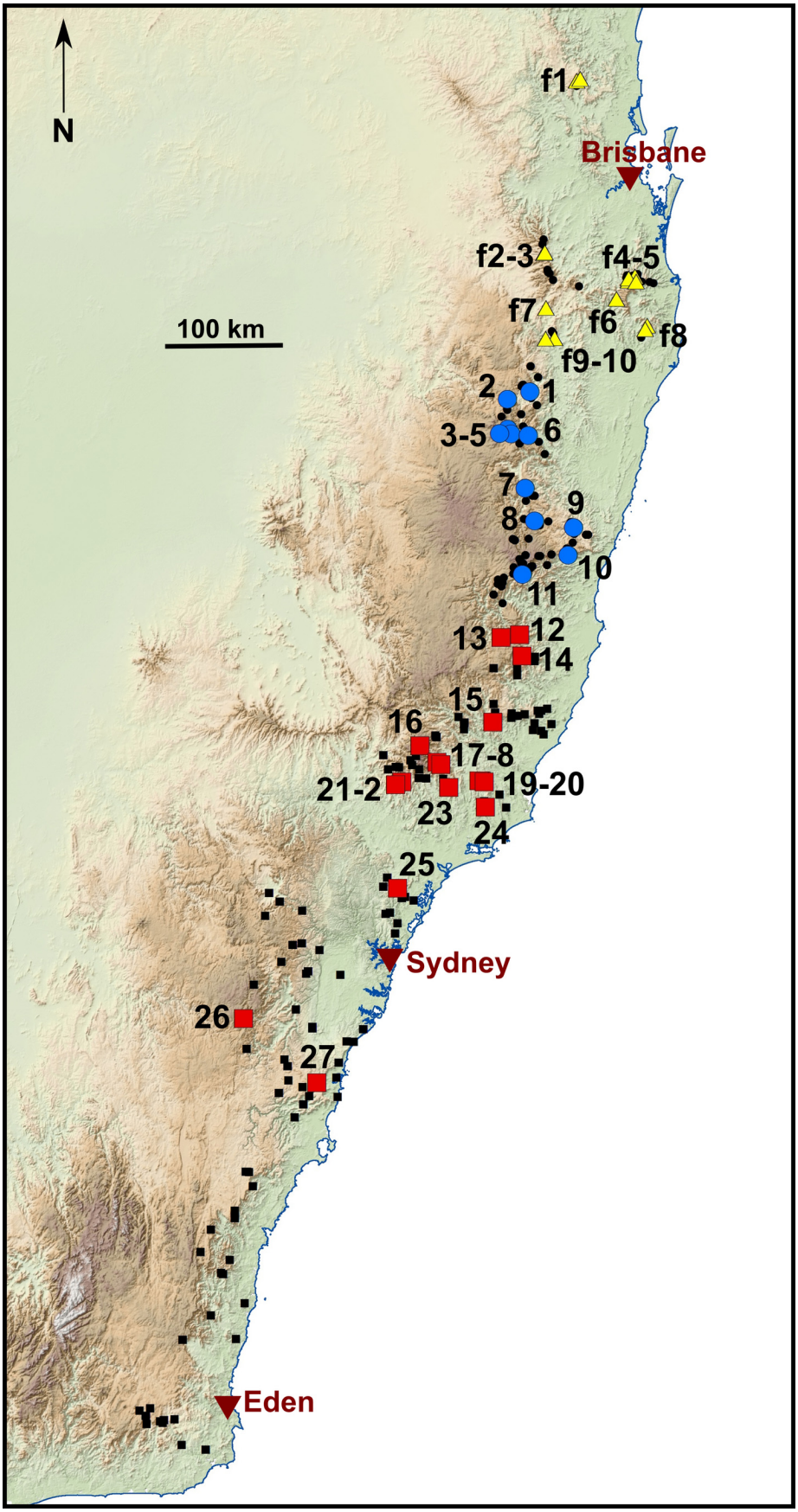

FIGURE 1. Map showing collection locations sampled for molecular genetic analysis of *Mixophyes* in eastern Australia. Alphanumeric codes refer to collection location details in Table 1. Small black dots are voucher records from the Atlas of Living Australia, yellow triangles—*M. fleayi*, blue circles—*M. balbus* northern group, red squares—*M. balbus* southern group.

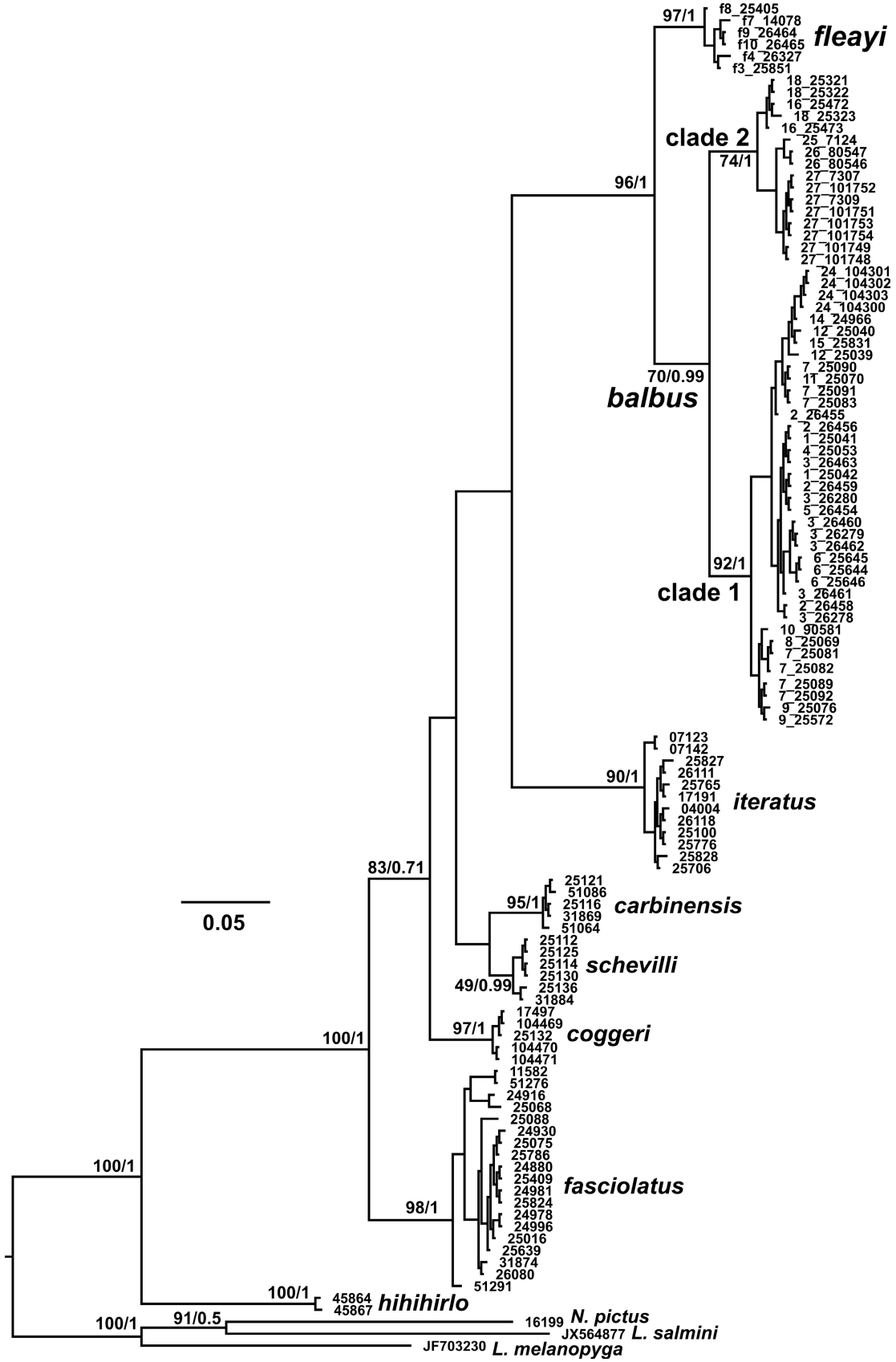

FIGURE 2. Bayesian phylogram of relationships among mitochondrial *ND2* haplotypes in *Mixophyes*. Values at nodes represent ML bootstrap proportions (left) and Bayesian posterior probabilities (right). The tree was rooted with sequences from the outgroups and *Lechriodus melanopyga*, *Limnodynastes salmini* and *Neobatrachus pictus* (Table 1). Alphanumeric codes refer to collection location details and ABTC numbers in Table 1.

Phylogenetic analyses. In both the IQTree and SVDQuartets phylogenetic analyses of the SNP data with *M. fasciolatus* as the outgroup, *M. fleayi* is a strongly supported sister lineage to *M. balbus* (Fig. 3). Two groups are present within *M. balbus*. The northern group comprises locations 2-11 and the southern group, comprises locations 12-27 with strong support in both the analyses for both lineages and the relationships amongst them.

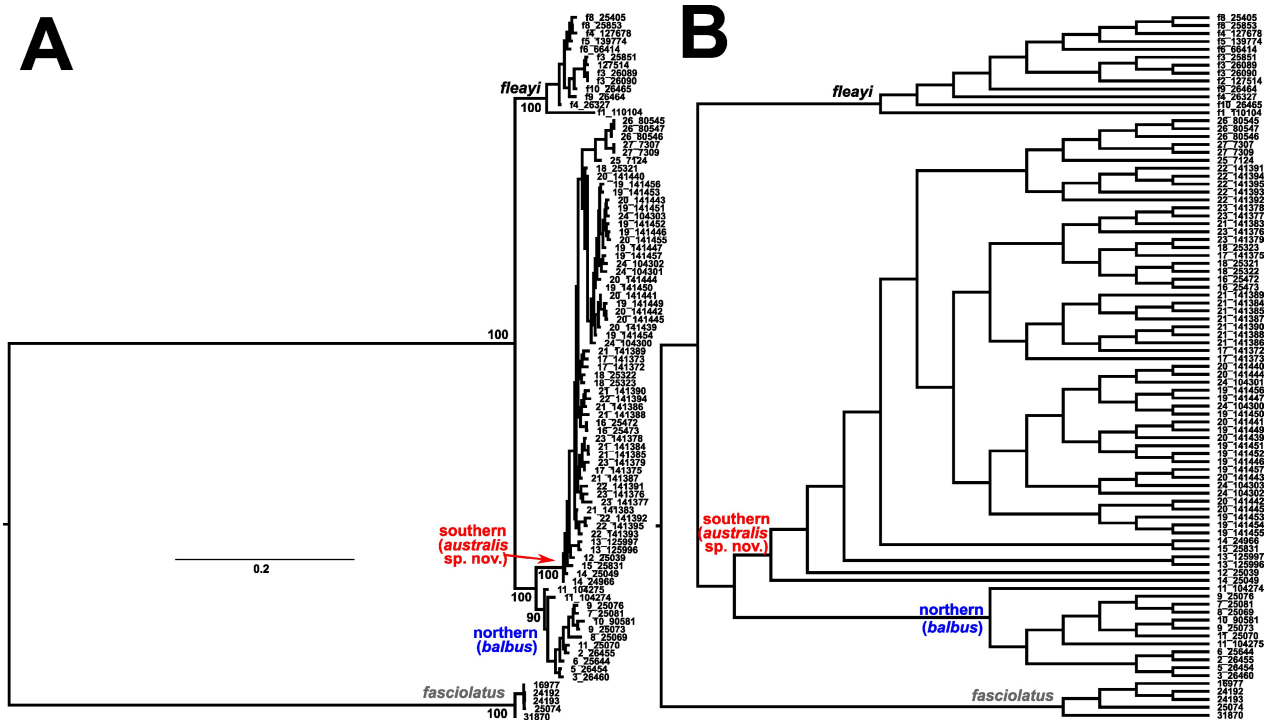

FIGURE 3. **A**) Maximum Likelihood phylogram and **B**) SVD Quartets cladogram of relationships among *Mixophyes* based on Single Nucleotide Polymorphisms. Alphanumeric codes refer to collection location details and ABTC numbers in Table 1.

Cluster analyses. Three clusters were apparent in the PCoA biplot of PC1 versus PC2 (Fig. 4A), corresponding to *M. fleayi* and the northern and southern groups within *M. balbus* that were delineated in the SNP phylogenetic analyses (Fig. 3). The northern group comprises samples from along the eastern uplands of the Great Dividing Range, from the New England Range in the north, south to the Gibraltar Range, then to the Dorrigo Plateau, and finally the Styx River. The southern group comprises samples from Carrai Plateau and Oxley Wild Rivers along the eastern uplands through the Barrington and Myall Ranges in the north, to the Central Coast Range and Blue Mountains, and the Illawarra escarpment in the south. The Macleay River seems to be the break between the two groups. The number of loci having a fixed difference between *M. fleayi* and the northern and southern groups were 599 and 980 respectively, while 198 loci showed a fixed difference between the northern and southern groups (Table 3). All three values were significant after simulation indicating that sampling error is not a likely explanation for the level of divergence observed (Table 3).

TABLE 3. Pairwise fixed difference analysis of the northern and southern group of *Mixophyes balbus* and *M. fleayi*. Upper matrix: number of loci showing a fixed difference, lower matrix: expected number of loci showing a fixed difference. Bolded values are significant after simulation. N is the number of individuals analysed.

	1	2	3
N	12	58	13
1—northern group (*M. balbus*)	-	**198**	**599**
2—southern group (*M. australis* **sp. nov.**)	38	-	**980**
3—*M. fleayi*	202	241	-

In the PCoA biplot of PC3 versus PC4 (Fig. 4B), four sub-clusters (S1-S4) were apparent among samples from the southern group, comprising locations: S1–12, 13, 14, 15; S2–16, 17, 18, 21, 22, 23; S3–19, 20, 24 and S4–25, 26, 27. Sub-cluster S4 was substantially divergent from the other three sub-clusters having a fixed difference at more

than 260 loci with each of the other three (Table 4). All three pairwise fixed difference values were significant after simulation indicating that sampling error is not a likely explanation for the level of divergence observed.

TABLE 4. Pairwise fixed difference analysis among *Mixophyes* southern genetic sub-clusters S1 to S4. Upper matrix: number of loci showing a fixed difference, lower matrix: expected number of loci showing a fixed difference. Bolded values are significant after simulation. N is the number of individuals analysed.

	S1	S2	S3	S4
N	6	25	22	4
S1	-	1	2	**263**
S2	4	-	0	**276**
S3	9	0.1	-	**432**
S4	258	96	152	-

In the STRUCTURE cluster analysis without the outgroup (Fig. 4C), the optimal value for k was three, comprising the three clusters (*M. fleayi*, and the northern and southern groups within *M. balbus*) that were observed also in the PCoA (Fig 4A). In the STRUCTURE cluster analysis of the southern group only, the optimal value for k was two based on the Delta K criterion of Evanno *et al.* (2005) and L(k), with the pattern of ancestry consistent with sub-cluster S4 being divergent from the other three sub-clusters as was observed in the PCoA (Fig 4D).

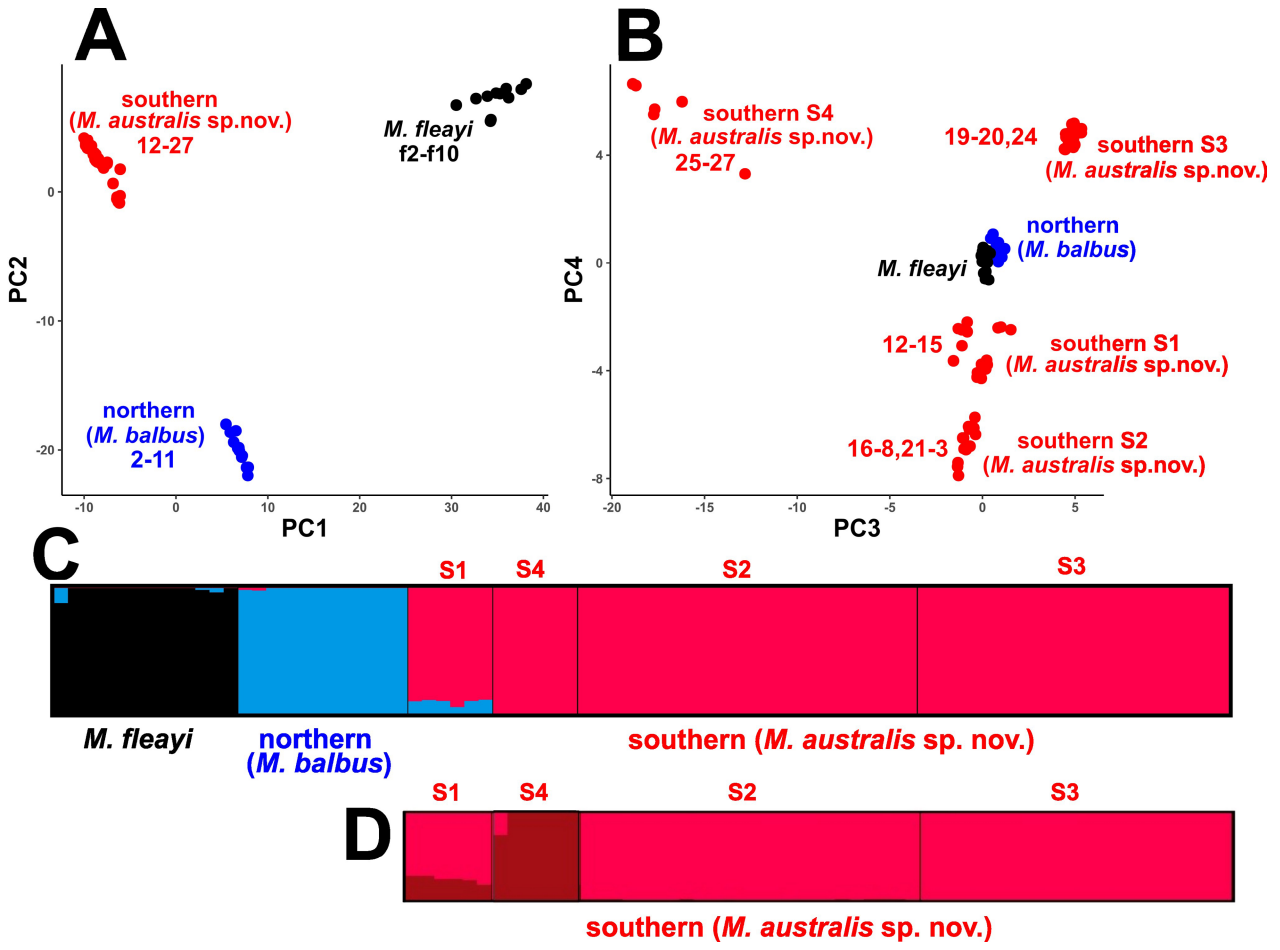

FIGURE 4. Single Nucleotide Polymorphism Principal Component Analysis of *Mixophyes fleayi* and *M. balbus*. **A**) PC1 v PC2, **B**) PC3 v PC4, **C**) STRUCTURE barplot of all ingroup samples, **D**) STRUCTURE barplot of southern group samples only.

Morphology. Morphometric analysis and inspection of the external morphology of genotyped adult specimens indicated that with a few exceptions, the northern and southern groups can be distinguished by a combination of morphological measurements, and patterns on the dorsal surface of the limbs (Figs. 5–8, Table 5).

TABLE 5. Mean (±SD) and ranges for 12 body measurements (see Material & Methods) and eight ratios of *Mixophyes balbus* and *M. australis* **sp. nov.** N—sample size.

	Males		Females	
Group	*balbus*	*australis*	*balbus*	*australis*
N	50	89	14	30
SVL	60.3±5.5	59.9±3.7	77.7±4.4	72.5±4.6
	49.3–70.6	52.3–69.1	65.9–83.2	63.7–81.4
HL	23.1±2.2	22.5±1.7	28.7±1.8	26.5±2.1
	19.1–26.7	19.8–26.9	23.8–31.2	23.1–30.9
HW	24.6±2.1	24.3±1.2	31.9±2.1	29.7±1.7
	20–28.7	20.9–26.9	26.5–34.3	26.9–34.2
TD	5.6±0.6	5.7±0.6	6.5±0.8	6.5±0.9
	4.4–6.9	4.6–7	4.9–7.5	5.0–8.3
ED	7.1±0.6	7.5±0.7	8.2±0.5	8.6±0.6
	5.6–8.2	5.7–9.3	7.3–8.9	7.2–9.8
EN	4.9±0.5	4.9±0.4	6.5±0.6	5.9±0.5
	4.0–5.9	3.7–5.9	5.7–7.7	5.1–7.0
IOD	10.8±1.2	10.5±1	13.7±0.9	12.7±1.1
	8.8–13.1	8.6–12.8	10.9–14.9	11.1–14.9
IND	5.7±0.6	6.2±0.7	7.2±0.7	7.2±0.8
	4.4–7.2	4.5–7.6	6.2–8.4	5.9–8.8
FLL	15.7±1.5	15.9±1	19.6±2.1	18.7±1.4
	12.9–19.2	12.7–17.8	13.7–22	16.9–22.1
Fin3L	15.6±1.7	15.1±1.3	20.5±1.8	18.5±1.5
	12.3–18.8	10.6–17.9	16.2–23.7	16–22.5
TL	36.1±3.5	37.2±1.9	46.8±3.1	44.2±2.6
	29.2–44.5	33.4–43	40.1–51.9	39.4–49.1
IMT	4.9±0.6	4.5±0.4	6±0.7	5.5±0.5
	3.9–6.1	3.6–5.7	4.5–6.8	4.8–6.4
HW/SVL	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0
	0.4–0.5	0.4–0.5	0.4–0.45	0.4–0.4
HL/SVL	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0
	0.3–0.4	0.3–0.4	0.4–0.4	0.3–0.4
HL/HW	0.9±0.0	0.9±0.1	0.9±0.1	0.9±0.1
	0.8–1.0	0.8–1.1	0.8–0.97	0.8–1.0
ED/EN	1.4±0.1	1.5±0.2	1.3±0.1	1.5±0.2
	1.2–1.7	1.1–2.0	1–1.46	1.1–1.8
EN/IOD	0.5±0.1	0.5±0.1	0.5±0	0.5±0.1
	0.4–0.6	0.3–0.6	0.4–0.53	0.4–0.6
ED/IND	1.3±0.1	1.2±0.2	0.9±0.1	0.8±0.1
	1.0–1.6	1–1.8	0.8–1.0	0.7–1.0
EN/IND	0.9±0.1	0.8±0.1	0.9±0.1	0.8±0.1
	0.7–1.1	0.6–1.2	0.8–1.0	0.7–1.0
TL/SVL	0.6±0.0	0.6±0.0	0.6±0.0	0.6±0.0
	0.6–0.7	0.6–0.7	0.6–0.65	0.6–0.7

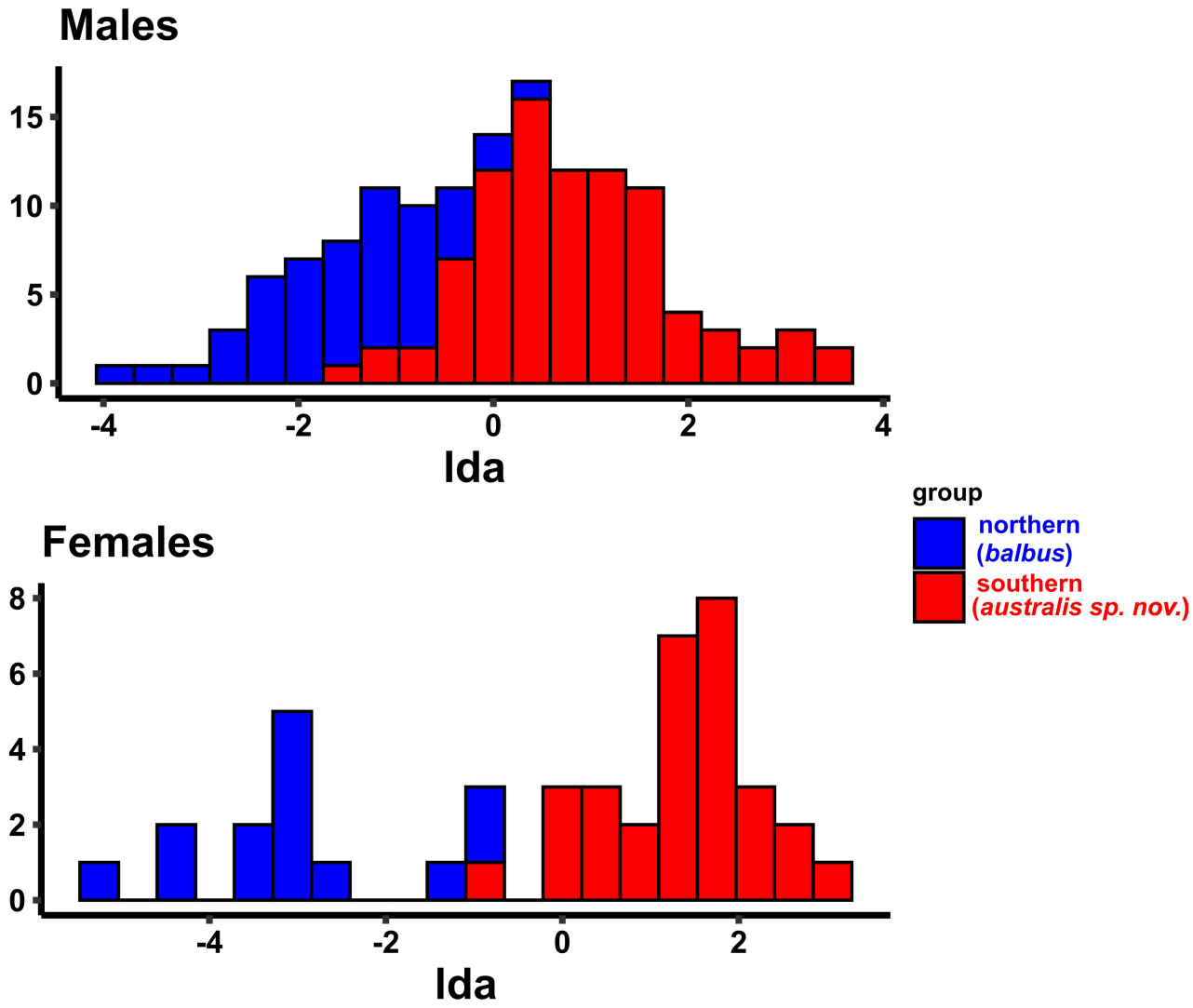

FIGURE 5. Plots of linear discriminants for analyses of morphological variation among *Mixophyes balbus* (blue) and *Mixophyes australis* **sp. nov.** (red).

Comparisons of SVL of sexes within each of the genetic groups showed that females are significantly larger than males (northern group, Z = -3.465, $P < 0.0001$; southern group, Z = -10.201, $P < 0.0001$). Adult females of the northern group are significantly ($p < 0.0001$) larger (mean 78.9 mm) than those of the southern group (mean 72.3 mm). However, the range of female SVL measurements overlap considerably and the body lengths of adult males are not significantly different so these measures alone cannot be used to distinguish between the two groups (Table 5). Three other measurements, arm and leg length and the length of the inner metatarsal tubercle, are also significantly different between the two groups (Table 5).

The pattern of cross bands on the dorsal surface of the limbs differs between the northern and southern groups (Fig. 6)**.** In the northern group, they comprise wide, highly contrasting bands that span the entire dorsal surface of the limb interspersed with narrower bands that only extend across the mid-dorsal surface of the limb. The wide bands are frequently expanded at their anterior and posterior ends. In contrast in the southern group, the bands are narrower and more uniform in width (Fig. 6).

Twenty five of 183 specimens (17 males and 8 females) did not conform to the diagnoses of the northern and southern groups on the basis of the limb cross bar pattern, (Supplementary Table S2). Of the six northern males only one, which had an intermediate phenotype, was found close to the range margin with the southern group, at the Styx River (location 11). This individual was not genotyped, but two other individuals from the same location, with conforming phenotype, were genotyped. For the 11 southern males none were from the range margin with the northern group. None of the 8 southern females were found close to the range margin with the northern group.

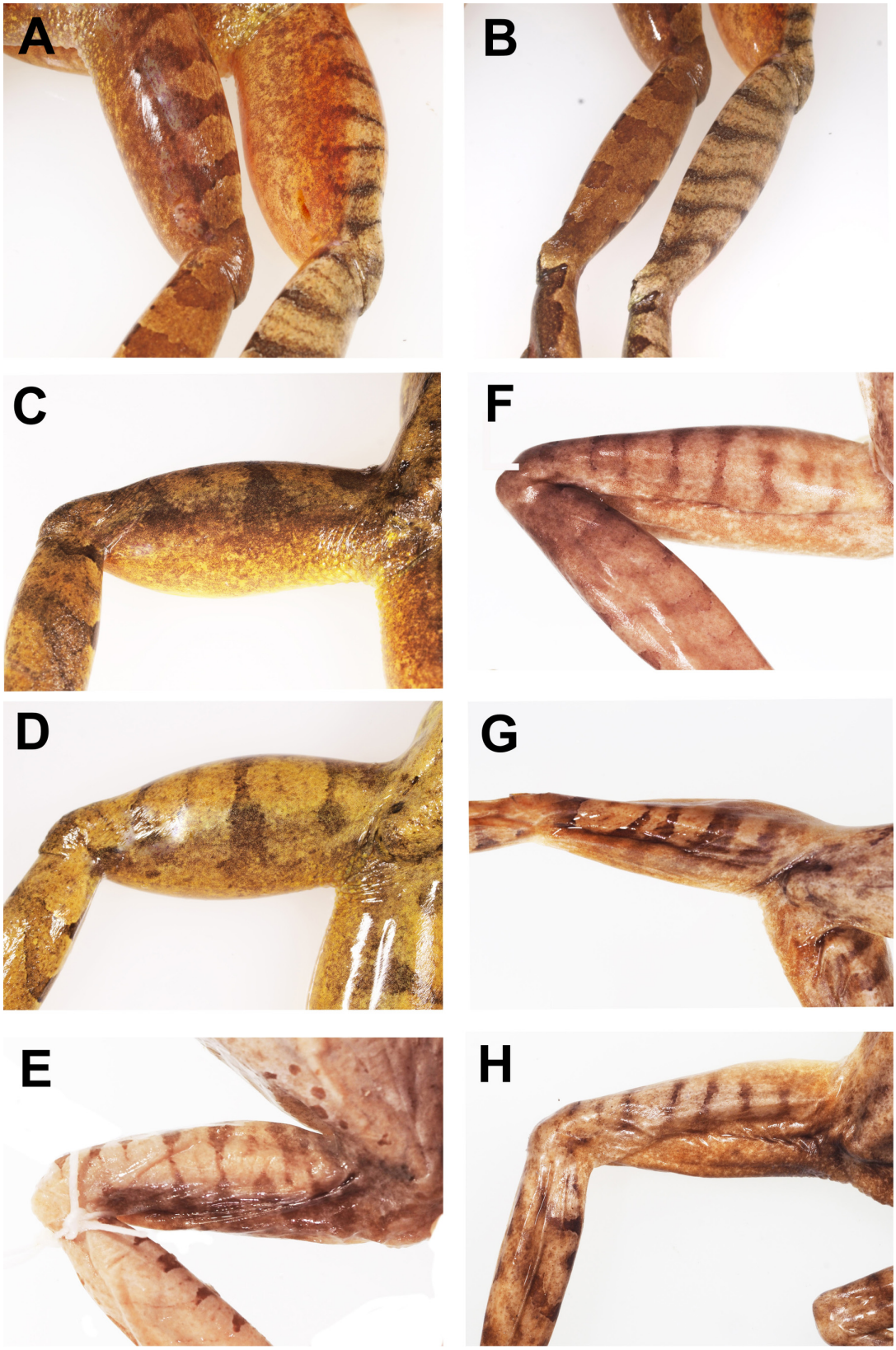

FIGURE 6. Cross bar patterns on hindlimbs of *Mixophyes*. Side by side comparison of **A)** thigh and **B)** tibia-fibula in life of left *Mixophyes balbus* from Coombadjha Creek Washpool NP and right *M. australis* **sp. nov.** from Congewai Creek, Watagan NP. Thighs of *Mixophyes balbus* from **C)** Little Styx River, New England NP, **D)** Thungutti Creek, New England NP, **E)** Coombadjha Creek, Washpool NP, and *Mixophyes australis* **sp. nov.** from **F)** Congewai Creek, Watagan NP, **G)** Frying Pan Creek, Chichester SF and **H)** Sharpes Creek, Gloucester Tops NP.

In a stepwise linear discriminant analysis (LDA) of 44 adult females (14 northern and 30 southern group individuals), 100% were correctly classified. The misclassification proportion after jackknifing was 91%. Two variables (HW, HL) had the highest contribution to the model for females (Supplementary Table S2).

In a stepwise LDA of 139 adult males (50 northern and 89 southern group), 12 (9%) were miss-assigned. Northern specimens were correctly assigned in 43 out of 50 cases (86%) and southern specimens were correctly assigned in 84 out of 89 cases (94%). The misclassification proportion after jackknifing was 86%. All but one of the specimens that were misclassified were located far from the range margin of the respective groups (Supplementary Table S2). The exception was a southern individual, SAMA R59916, that was SNP genotyped, from Dingo State Forest (location 15) which is 130 km from the nearest northern location (location 11). Two variables (TL, HW) had the highest contribution to the model for males (Supplementary Table S2).

Reproductive call. The reproductive call in both the northern and southern groups comprises a series of advertisement calls without regular inter-call intervals, that may be followed or interspersed with one or more encounter calls, dependent on the behavioural context (Fig. 9A, B).

Advertisement and encounter calls can be distinguished by ear and by their pulse structure. The advertisement call sounds like a rapid "krook..krook..krook.…kra-a-ak..kruk..kruk" and the encounter call a harsh "wark". The advertisement call is classified in the pulse repetition category and the encounter call in the pulsatile sound category of Beeman (1998). The advertisement call is one or more (commonly two) notes with short fully amplitude modulated pulses, and the encounter call is a single note with dense pulses, contrast Figs 9C with 9E and 9D with 9F. Advertisement and encounter calls have similar dominant frequencies.

The overall structure of the advertisement calls of both groups are similar with calls comprising one or more (commonly two) notes (Fig. 9C, D). Note duration is less than 1 s in both groups (Table 2) and comprises rapidly repeated pulses that are fully modulated (Fig. 9C, D). The number of notes produced depends on the behavioural context, such that when males are calling independently the call is often only one or two notes, but when calling in a chorus, males often produce calls that may extend up to five notes. Compared to the first note, the second and subsequent notes differ in the number and temporal arrangement of pulses (Fig. 9C, D). Pulses within the note are produced singly at the start and then in groups of two and occasional three pulses with decreasing inter-pulse duration towards the end of the note (Fig. 9C, D). There is no evidence of amplitude modulation in the call, with notes showing a spike shape in the waveform (rapid rise and rapid decay in amplitude of individual pulses) and the energy of each note being approximately equal (Fig. 9C, D). Pulse structure of individual notes shows a symmetrical rise and fall in the energy (Fig. 9C, D).

Males calling in choruses occasionally produce a long series of notes with single pulses, at a longer inter-note interval than in the advertisement call described above. The function of the single pulsed notes is not known, and we have not included them in our quantitative analyses below.

The overall structure of the encounter calls in both groups is similar with calls lasting 0.20–0.29 s and comprising two or three initial pulses that are fully amplitude modulated followed by a group of dense pulses lasting 0.20–0.26 s that are not fully amplitude modulated (Fig. 9E, F). Amplitude varies across the dense pulses starting high, decreasing in the middle, and gradually rising again prior to an abrupt cessation (Fig. 9E, F). Dominant frequency is similar to that of the advertisement call and there is no frequency modulation (Fig. 9E, F).

While the overall structure of advertisement and encounter calls in the northern and southern groups is similar, there are consistent quantitative differences in the number of notes and dominant frequencies of advertisement calls (Table 2). Following correction for temperature the note duration (first and second note of the advertisement call) was not significantly different between the northern and southern groups. While the duration of notes is similar, the number of pulses in notes produced by the southern group is approximately two-thirds that of the northern group, and they have a longer inter-pulse duration (Table 2). The dominant frequency of the calls is slightly different, with the mean of the first and second note 817.5 Hz and 758.2 Hz in the northern group, and 731 Hz and 741.7 Hz, in the southern group (Table 2). Call trait values reported by Straughan (1966) from the type locality which included the holotype of *M. balbus* were within the range of other northern group populations in temporal features, but the dominant frequency was greater than we observed (mean 950 compared to 817.5 Hz) (Table 2). The advertisement calls of one individual recorded by Dr M Littlejohn in east Gippsland in 1965 (Littlejohn 1969), are typical of those of other southern group populations.

Systematic Implications. Our analyses of thousands of nuclear gene SNPs revealed the presence of two deeply divergent genetic groups in *M. balbus*. The presence of many loci showing a 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 a separate species (Georges *et al.* 2018). The closest samples of each group (locations 11 and 12) are separated by 51 km. Furthermore, multivariate morphological comparisons show the two groups differ in body proportions with 91% of male and 100% of female individuals being correctly classified in LDA. The two groups differ in adult size and the number and pattern of cross bands on their dorsal surfaces of the legs and thighs. The advertisement calls differ in the number of pulses in the note, but we have not determined if these differences lead to assortative mating in relation to genetic ancestry by laboratory testing and we have not found any locations where the groups come into contact.

Our data demonstrate a marked discordance between the geographic distribution of mitochondrial clades and the nuclear genetic lineages (Fig. 10). In our nuclear data the Macleay River Valley marks the adjacent range margins of the northern and southern groups (between locations 16 & 18), whereas the boundary between the two major mitochondrial clades is well within the distribution of the southern group (between locations 15 & 24). We also note that, in the southern group, the distribution of mitochondrial clade 2 does not coincide with the deepest subdivision in that group, i.e., sub-cluster S4 versus sub-clusters S1–3 (Fig. 4B, D) as clade 2 includes localities from sub-clusters S2 and S4. Discordance between mitochondrial DNA and nuclear genes (or nuclear gene encoded phenotypes) can result from introgression of mtDNA between closely related lineages without any evidence of substantial gene flow between nuclear genomes (Sloan *et al.* 2017, Toews & Brelsford 2012). This finding which occurs in our data and as in many other examples in the recent literature (Kehlmaier *et al.* 2019, Mahony *et al.* 2022), does not impinge on the species status of the taxa involved.

The type locality of *M. balbus* Straughan is Point Lookout, New England National Park, NSW, which is deep within the range of the northern group, and the morphology of adults, including the holotype (AMS R. 25922), and the advertisement call from this locality are consistent with our new northern group samples.

We have not been able to genetically confirm the taxonomic status of populations south of the Illawarra Escarpment, which may be extinct, as material for genetic analysis is presently not available. We also lack advertisment calls for animals south of the Central Coast Range, except for historic recordings from the Gippsland area (Littlejohn 1969, 1987). Given the similarity in call structure of Gippsland animals to that of our 'southern' group (Littlejohn 1969), we conservatively assign these populations to the southern group which we name as a new species.

Systematics

Mixophyes Günther, 1864

Species of *Mixophyes* are distinguishable from all other Australian frogs by the following unique combination of characters: pupil vertical when constricted; vomerine teeth in front of choanae, tongue round, one quarter to one fifth free at posterior margin; tympanum distinct, oval; limbs with dark cross bands; fingers and toes long, without expanded terminal disc; fingers without webbing, toes with well-developed webbing; amplexus axillary; eggs fertilized in water and non-foamy egg mass either deposited among pebbles or debris or onto bedrock in slow flowing water in *M. balbus, M. australis* **sp. nov.** and *M. fleayi,* or eggs flicked by the females foot out of water and adhere on bank or overhang above water in *M. fasciolatus, M. iteratus, M. coggeri* and *M. schevilli* (Straughan 1968, Hoskin 2010, Anstis 2013, Knowles *et al.* 2015).

Mixophyes balbus Straughan, 1968.

Northern Stuttering Frog
(Figs 6, 7, 11)

Holotype. AMS R.25922, a male collected from Point Lookout, New England National Park, New South Wales, between 4,250 and 4,750 feet altitude on 15 October 1965.

Dimensions of holotype (in mm). SVL, 70; TL, 43; HL, 27; HW, 27; EN, 6; IND, 6; ED, 8; IOD, 17; TD, 7; FLL, 19; Fin3L, 14; MT, 6. Straughan (1968) recorded the SVL of the holotype as 75 mm. We are unable to explain this discrepancy other than measurement error by Straughan and note that none of the males that we measured had

an SVL >70.6 mm (Supplementary Table S2). Straughan (1968) did not provide any other measurements of the holotype.

Material Examined. Vouchers examined for morphological analyses are listed in Supplementary Table S2.

Diagnosis. *Mixophyes balbus* is distinguished from *M. hihihorlo* by a interrupted vertebral stripe (vs. uninterrupted) and presence of dark triangular patch on upper lip in front of nostril (vs. absent); from *M. iteratu*s and *M. carbinensis* by absence of black colouration with numerous small rounded pale spots on posterior surface of thigh (vs. light brown without any pale spots); from *M. coggeri* by having posterior of thigh uniform (vs. with irregular pale blotches); from *M. schevilli* by having a discontinuous (vs. continuous) mid-dorsal stripe; from *M. fleayi* and *M. fasciolatus* by lack of prominent black spots on flanks and further from *M. fasciolatus* by occurrence of pigmented patches on maxilla; from *M. australis* **sp. nov.** by having five wider and more diffusely marked cross bands on thigh (vs. up to ten thin dark cross band in *M. australis* **sp. nov.)**; male advertisement call comprises one to four notes with mean number of pulses in first and second notes being more than in *M. australis* **sp. nov.** (mean 12.24 vs. 7.24) with more double pulses in these notes.

In the field *M. balbus* is most likely to be confused with *M. fasciolatus* and *M. iteratus* which occur sympatrically. In addition to the features listed above, *M. balbus* can be distinguished from *M. iteratus* by the webbing on the toes reaching only to the second joint on the third toe, compared to distal joint in *M. iteratus*; from *M. fasciolatus* by the occurrence of a metallic silver-blue crescent in the top quarter of the iris (vs. absent in *M. fasciolatus*), and the absence of sharply demarcated cross bands on the limbs that form distinct triangular markings on the posterior surfaces of the arms and legs (vs. presence in *M. fasciolatus*).

Description including variation. Straughan's (1966, 1968) description of variation in *M. balbus* included material from the southern group which we show is a new species. Here we provide detailed images of the type (Fig. 11) and a revised description of variation in *M. balbus* that excludes material from the range of the new species.

A moderate sized frog, sexually dimorphic, females larger (up to 83 mm SVL) than males (up to 71 mm SVL). Females are an average of 1.2 times the SVL of males (Table 5), but they are otherwise similar in body proportions (Table 5), colour in preservative and patterning.

Tympanum large, conspicuous (TD/ED mean = 0.79±0.07, 0.62–0.96), oval shaped with longer axis vertical. Eyes prominent, pupil vertical, iris in life silver-blue or brown above, brown below. Head flattened, length slightly shorter than or equal to width (HL/HW mean = 0.93±0.05, 0.70–1.03); snout prominent (ED/EN mean = 1.4±0.1, 1.2–1.7), gently rounded when viewed from above and in profile; nostrils more lateral than superior; canthus rostralis well defined, slightly concave; loreal region nearly flat, inclined laterally; eye to naris distance slightly smaller than or approximately equal to internarial span (EN/IND mean = 0.88±0.1, 0.71–1.13). Vomerine teeth relatively long, oblique rows angled from anterior margin of choanae to midline between choanae. Tongue approximately rectangular, not notched posteriorly. Dorsum smooth or finely granular, belly smooth.

Hind limbs long and robust (TL/SVL mean = 0.6±0.02, 0.55–0.65).

Fingers moderately long, robust, unwebbed, relative length 3>2=4=1. Subarticular and palmar tubercles prominent. Finely granular brown nuptial pad on first and second finger and extends ventrally to the inner palmar tubercle in breeding males.

Toes moderately webbed; on outer side of toes webbing reaches penultimate subarticular tubercle on toes 1 and 2, penultimate tubercle on toe 3, and second subarticular tubercle on toe 4. Tips of toes slightly expanded but lacking obvious discs (Fig. 11). Relative lengths of toes 4>5>3>2>1. Prominent round or oval conical sub-articular tubercles present; outer metatarsal tubercle absent; inner metatarsal tubercle prominent relatively straight, with well-developed outer edge, approximately equal to length of toe 1, not sharp or keratinized, pigmentation same as plantar surface.

Colour and Pattern in Life. Dorsal surfaces rich copper to tan or dark brown, sometimes with burnt orange or pink wash towards groin; a posteriorly oriented v-shaped inter-orbital bar that extends as a mid-dorsal stripe, finely edged in black and highly variable in length and shape with uneven edges terminating in mid-sacral region; remainder of dorsal surface either sparsely flecked with small black spots or a few larger blotches but not both (Fig. 7). A dark triangular patch on either side of snout with apex at external nostril does not incorporate area immediately surrounding nostril. A dark lateral head-stripe extends from slightly posterior of nostril, through eye, curves over tympanum, and extends posteriorly down anterior margin of tympanum, sometimes including upper third of tympanum, terminating above axilla; width variable but in all cases posterior margin is widest. Loreal region light-brown coloured with darker patches along upper jaw (maxilla).

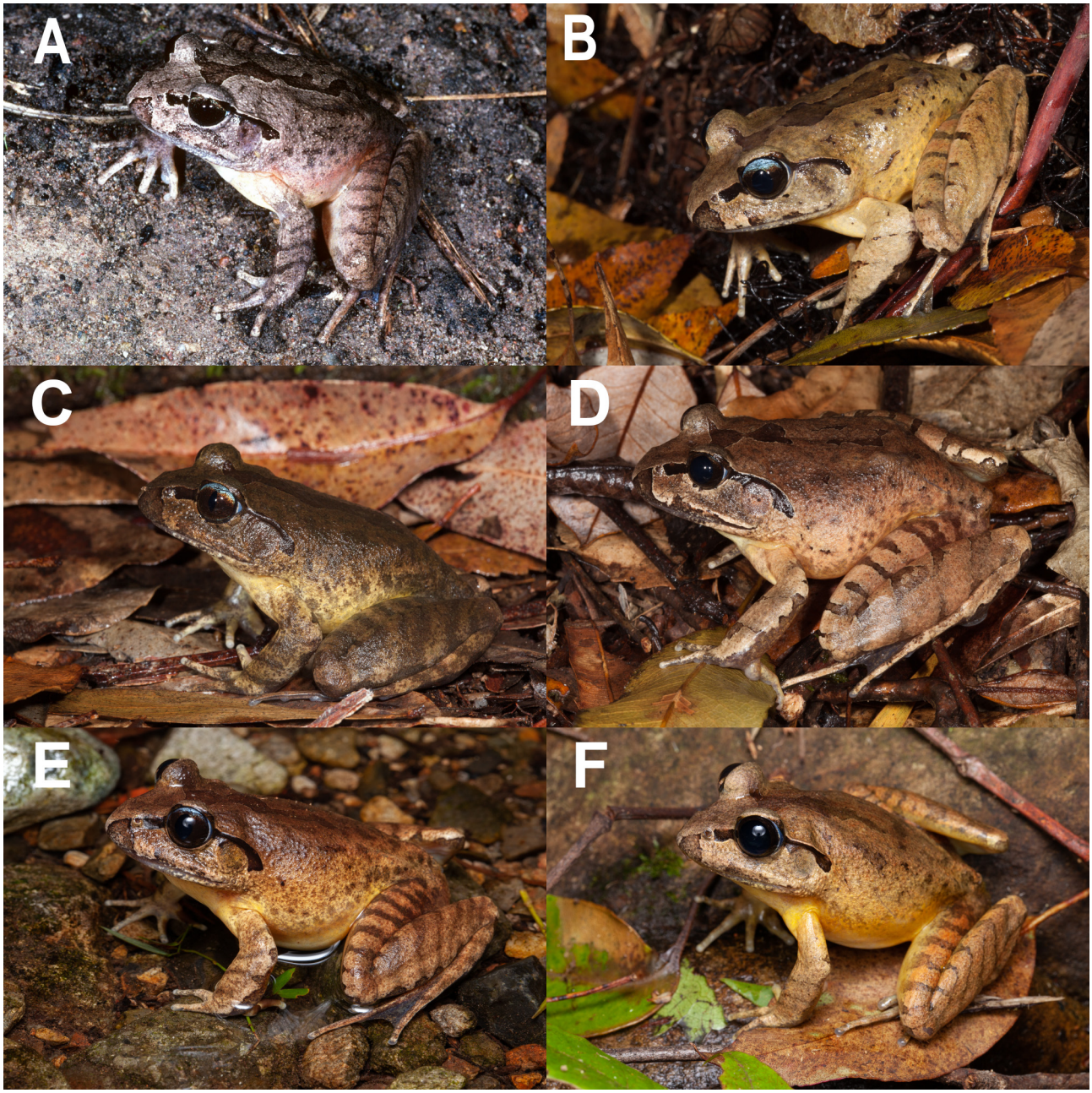

FIGURE 7. *Mixophyes balbus* in life. **A**) Rockadooie Ck, Curramore SF, Michael Mahony, **B**) Coombadjha Ck, Washpool NP, Stephen Mahony, **C**) Washpool NP, Gibraltar Range, Stephen Mahony, **D**) Coombadjha Ck, Washpool NP, Stephen Mahony, **E**) Forestland SF, Stephen Mahony, **F**) Forestland SF, Stephen Mahony.

Lateral surfaces and groin flushed with reddish tan or peach, without spots or blotches, these surfaces noticeably lighter than dorsum, with colour diffusing gradually into ventral colouration. Venter including throat and ventral surface of thighs immaculate white, cream or with light lemon-yellow wash; lower jaw edged with diffuse brown wash.

In adults, iris dark brown merging into golden brown above pupil with brown to iridescent pale blue dorsal crescent. Upper third of iris in juveniles may be as above or flame orange.

Upper surfaces of legs, feet, arms and hands with dark transverse cross bars, not extending on to posterior thighs; posterior surface of thighs uniform brown to burnt orange brown and slightly mottled; anterior lateral edge of shank, posterior lateral edge of arms, and plantar surface of feet and of tarsal surface dark brown; lateral palmar surfaces of outer fingers dark brown to black.

Reproductive call. The male advertisement call comprises one to four short notes repeated in rapid succession.

Because of the variation in the number of notes in the call we provide means and ranges for measurements of call traits for the first two notes. Mean duration for a two note call is 0.45 s (range 0.45–0.48 s). The first note is of longer duration than the second (means 0.68 and 0.41 s, ranges 0.39–0.96 s, and 0.28–0.63 s respectively), with a short inter-note interval (mean 0.36 s), and with more pulses in the first than the second note (mean 12.8, and 9.1, ranges 9–17 and 3–12 respectively). Pulse duration is much shorter (mean 0.015 s, range 0.014–0.016 s) than inter-pulse interval (0.083 s, range 0.081–0.084 s). Pulse structure varies within notes, with single pulses produced initially, followed by a series of pulses produced in couplets (Table 2, Fig. 9). The second note has more couplets than the first note (Fig. 9). Pulse repetition rate in the first note is lower than the second note (16.1 pps vs 21.0 pps) (Table 2). Mean inter-call interval is 4 s (range 3–8 s) and males usually produce a series of calls before a period in which all calling ceases. Calling occurs during the spring and summer seasons when climatic conditions are suitable (MM pers. obs. Details of suitable conditions not reported here). Within the calling season, calling is initiated by a male, and this stimulates calling in nearby males and a chorus of calls with a short lag time or small overlap between the call of one male and a nearby male occurs. The male encounter call which is a single note of moderate duration (mean 0.23 s, range 0.24–0.48 s) is often uttered among advertisement calls and males may produce several encounter calls in succession (Fig. 9C). The dominant frequency of the notes in the advertisement call differs slightly with the first note having a higher frequency than the second (817 Hz and 758 Hz, ranges 709–782, 659–961 Hz respectively). The dominant frequency of the encounter call (788 Hz, range 709 and 792Hz) is higher than the advertisement call and shows an initial narrow band frequency that expands to a broadband pattern. Advertisement and encounter calls are not frequency modulated. Representative call recordings have been deposited with the Australian Museum as multimedia record AMS R.188750.

Distribution. *Mixophyes balbus* occurs in drainages that flow to the east of the Great Dividing Range from the upper reaches of the Timbarra River catchment in north-eastern NSW, south to the northern catchments of the Macleay River (Fig. 1). The majority of records occur between 600–1500 m asl, and the species occurs entirely within the upper North Coast Bioregion (Thackway & Cresswell 1995).

Habitat and Ecology. Statistical modelling using presence and absence data and 24 environmental predictors sampled throughout the range of *M. balbus* and for northern populations of *M. australis* **sp. nov.**, showed a preference for the interiors of large forest tracts in areas with relatively cool mean annual temperatures, at sites that were typically free from any disturbance with a thick canopy and relatively simple understorey (NSW NEFBS 1994). Tracking studies indicate that the adults spend the majority of their life in the riparian zone (Lemckert & Morse 1999), and adults are observed rarely in terrestrial survey transects away from the riparian zone (Knowles *et al.* 2015) but are sometimes observed on roads near riparian zones.

Eggs and larvae. Embryonic development and larval morphology were described by Anstis (2013) from specimens collected at five locations that include two within the range of *M. balbus* (near Point Lookout, New England Range, Coombadjha Creek, Gibraltar Range) and three from within the range of *M. australis* **sp. nov.** (Tirrill Creek, Bulga State Forest, Sharpes Creek, Barrington Range, Gap Creek, Central Coast Range). No mention was made of morphological variation among the sites sampled that would indicate a difference between those from what is now known as *M. balbus* and *M. australis* **sp. nov.** (Anstis 2013), but reanalysis of these data based on our refined taxonomy is required. Larval morphology was described for specimens from Point Lookout NSW (type location) by Watson & Martin (1973) and are the same as described by Anstis (2013). Knowles *et al.* (2015) did not observe any differences in egg deposition mode at four locations for *M. australis* **sp. nov**. and one for *M. balbus*.

Assessment of IUCN threat category for *Mixophyes balbus.* Following our systematic findings and taxonomic actions herein, it is necessary to assess the IUCN threat categories for *M. balbus* and *M. australis* **sp. nov.** because previous conservation assessments were based on the presumption of a single species (Supplementary Text).

Based on the IUCN Red List criteria (IUCN 2019) and with information presented in other recent assessments (Gillespie *et al.* 2020, Geyle *et al.* 2021), we assess *M. balbus* to be Endangered, under category B1(a)(c) (Supplementary Text). The measured EOO (alpha-hull) is 1,477 km^2, which is well below the threshold score of <5000 km^2, and the distribution is fragmented (occurring in <5 locations). The Area of Occupancy (AOO) in the decade 2010–2020 is 880 km^2, which is well below the threshold level of 2000 km^2 for Endangered but above Critically Endangered (500 km^2) (Supplementary Text). While observed occupancy (EOO and AOO) place this species in the Endangered category, mapping of observation records for the past two decades shows no evidence of a continued decline in occupancy that can be inferred or projected, rather the occupancy has apparently stabilised during this time period, and the EOO and AOO have remained constant in the past two decades (Supplementary Text

Fig. S4). Field work indicates that the species can be found in each of the five locations, and there is no evidence of population reduction at these locations based on mapping of records over three generations (Supplementary Text Fig. S1). There is evidence that declines occurred at many sites prior to 2000 (NSW NEFBS 1994), but after this time there is evidence of a gradual recovery at many sites apart from previously occupied high elevation sites, and the species occurs across its historic latitudinal range (Mahony 2007, 2013).

A direct threat to *M. balbus* is the amphibian disease chytridiomycosis (Scheele *et al.* 2021). The available evidence is that *M. balbus* is an example of a species that is susceptible to chytridiomycosis, and that population declines can be precipitous at times when climatic conditions are suitable for disease transmission and progression. The observation of gradual recovery in population abundance and distribution in the past two decades indicates that some process of selective adaptation to combat the disease may be occurring and this provides several options for conservation recovery actions.

The indirect effects of climate change such as drought and wildfires may also impact on the species. The extent of fragmentation and isolation may be increased by landscape scale catastrophic wildfires (Legge *et al.* 2022), which may exacerbate local and regional declines. However, an assessment of vulnerabilities to wildfires based on biological traits indicated some level of resilience because of the species' burrowing behaviour and restriction to moist riparian habitats (Mahony *et al.* 2022). To understand and mitigate these threats we recommend that systematic monitoring be undertaken since there is good evidence of rapid declines in this species in the past.

***Mixophyes australis* sp. nov.**
Southern Stuttering Frog
(Figs 6, 8, 12)

Holotype. AMS R.188750 (ABTC 141374), adult male from Sharpes Creek, Gloucester Tops National Park, New South Wales, Australia, 32° 05' S, 151° 68' E, collected by Michael Mahony and Ross Knowles on 15 December 2015.

Material examined. see Supplementary Table S1.

Diagnosis. *Mixophyes australis* **sp. nov.** is distinguished from *M. hihihorlo* by the presence of a dark triangular patch on the upper lip in front of the nostril with its apex at the nostril (vs absence), and absence of an uninterrupted narrow vertebral stripe extending from between the eyes to just above the vent (vs. presence); from *M. iteratus* and *M. carbinensis* by absence of black colouration with numerous small rounded pale spots on the posterior surface of the thigh; from *M. coggeri* by having posterior of thigh uniform (vs. with irregular pale blotches); from *M. schevilli* by having a discontinuous (vs. continuous) mid-dorsal stripe; from *M. fleayi* and *M. fasciolatus* by the lack of prominent black spots on the flanks and further from *M. fasciolatus* by the occurrence of pigmented patches on maxilla; from *M. balbus* by the occurrence of up to ten thin dark cross bands on the thighs (vs. five wider and more diffusely pigmented cross bands in *M. balbus)*; male advertisement call comprises one to four phrases with the mean number of notes in the first and second phrase fewer than in *M. balbus* (mean 7.24 vs. 12.24) with fewer double pulses in these phrases; and with a lower dominant frequency in both the advertisement (752 Hz vs. 773 Hz) and encounter (704 Hz vs. 788 Hz) calls.

In the field, *M. australis* **sp. nov.** is most likely to be confused with specimens of *M. fasciolatus* and *M. iteratus* which occur sympatrically. In addition to the features listed above, *M. australis* **sp. nov.** can be distinguished from *M. iteratus* by less extensive webbing of the toes that reaches to the distal tubercle of the fourth toe in *M. iteratus*. From *M. fasciolatus* it can be distinguished by the occurrence of darker markings on the lateral margins of the maxilla and mandible, in *M. fasciolatus* the maxilla is unmarked and is lighter coloured than the general background producing a distinctive paler area; and the soles of the feet are not pigmented black versus pigmented brown to black in *M. fasciolatus*.

Dimensions of holotype (in mm). SVL, 67; TL, 39.6; HL, 26.9; HW, 26.2; EN, 4.9; IND, 7.1; ED, 8.1; IOD, 12.8; TD, 5.9; FLL, 16.7; Fin3L, 16.1; MT, 4.8.

Description of holotype. Head flattened, snout prominent, gently rounded when viewed from above and in profile; nostrils more lateral than superior; canthus rostralis well defined, slightly concave; loreal region nearly flat, inclined laterally (Fig. 12). Eye relatively large (ED/EN = 1.67), pupil vertical when constricted; eye to naris span longer than internarial span (EN/IND = 0.69); tympanum large, nearly three-quarters eye diameter (TD/ED = 0.73),

conspicuous, oval with vertical long axis. Vomerine teeth relatively long, oblique rows angled from anterior margin of choanae to midline between choanae; tongue approximately rectangular, not notched posteriorly.

Fingers robust, unwebbed. Subarticular and palmar tubercles prominent; relative length of fingers 3>2=4=1.

Hind limbs long (TL/SVL = 0.59); toes moderately webbed, on outer side of toes webbing reaches penultimate sub-articular tubercle on toes 1, 2 and 3, and second tubercle on toe 4, all toes with lateral flanges extending along toes to the terminal disc; tips of toes slightly expanded but not forming terminal disc (Fig. 12). Subarticular tubercles prominent, oval and conical; outer metatarsal tubercle absent; inner metatarsal tubercle prominent, slightly curved, with well-developed outer edge, approximately equal to the length of toe 1, not sharp or keratinized pigmented same as ventral surface of foot; relative length of toes 4>5>3>2>1.

Dorsum finely granular with small tubercules, venter smooth. Series of raised conical tubercles on ventral surface of thigh form a triangular area in pelvic patch with the tubercles becoming larger toward the mid-line and cloaca. Several large conical tubercles around the cloaca opening.

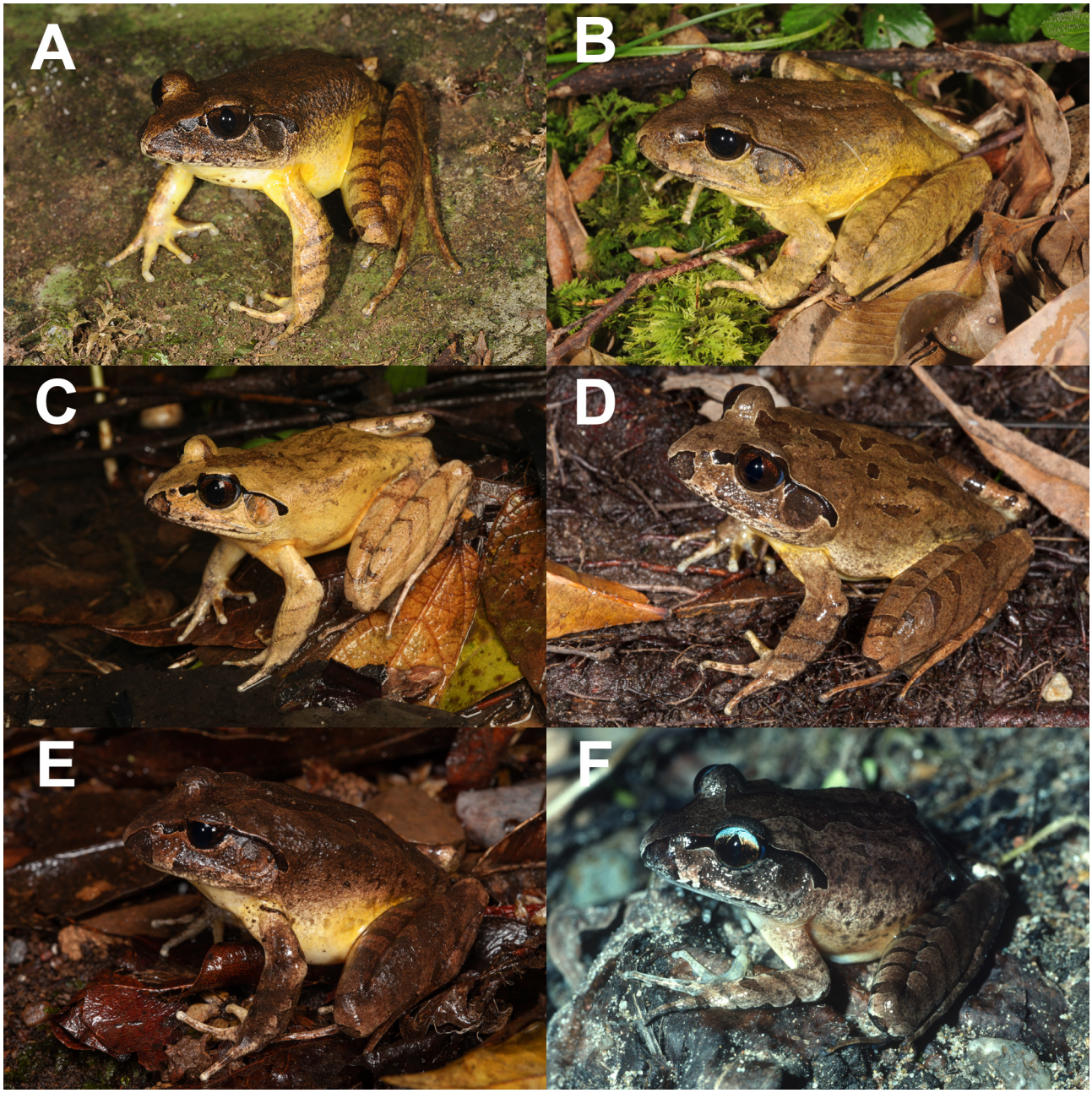

FIGURE 8. *Mixophyes australis* **sp. nov.** in life. **A)** Holotype AMS R188750, Sharpes Creek, Gloucester Top NP, Ross Knowles, **B)** Sharpes Creek, Gloucester Top NP, **C)** Gap Creek, Watagan NP, Stephen Mahony, **D)** Abbots Ck, Olney SF, Stephen Mahony, **E)** Cockerawombeeba Ck, Hastings Range, Michael Mahony, **F)** Cockerawombeeba Ck, Hastings Range, Michael Mahony.

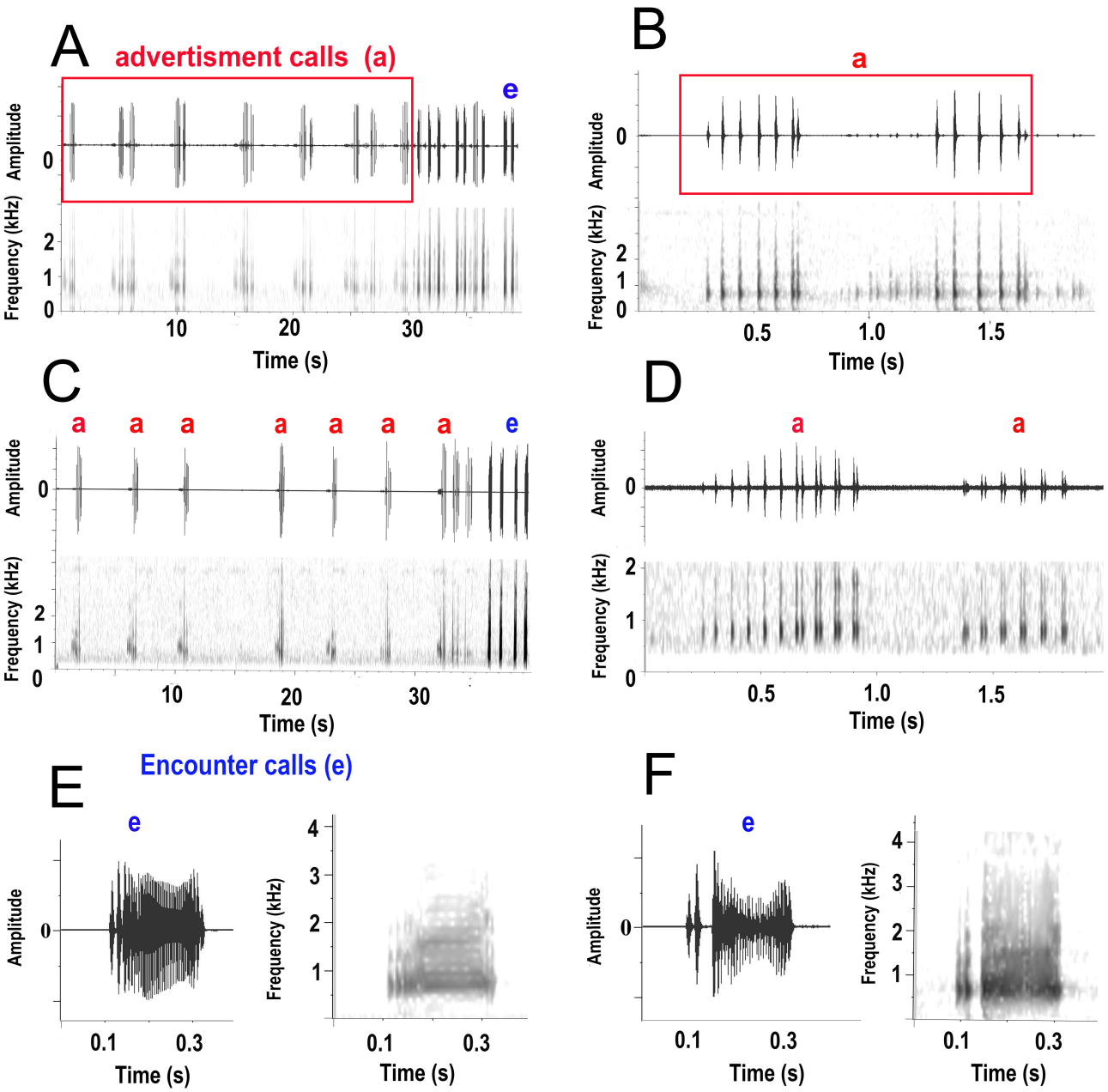

FIGURE 9. Reproductive calls of *Mixophyes balbus* and *Mixophyes australis* **sp. nov. A**) Oscillogram and spectrogram of a series of advertisement calls and encounter calls of *Mixophyes australis* **sp. nov.** over 40 seconds duration, **B)** a single call with the time axis expanded (1.5 seconds duration), **C**) oscillogram and spectrogram of a series of advertisement calls and encounter calls of *Mixophyes balbus* over 40 seconds duration, **D)** a single call with the time axis expanded (1.5 seconds duration), **E**) oscillogram and spectrogram of an encounter call of *Mixophyes australis* **sp. nov.**, (0.4 second time axis), and **F**) oscillogram and spectrogram of an encounter call *Mixophyes balbus* (0.4 second time axis). a = advertisement call, e = encounter call, and kHz is kilohertz.

Variation. SVL of males 52–69 mm and females 64–81 mm (Table 5). Females are larger than males, being an average of 1.3 times the SVL of males (Table 5) but are otherwise similar in body proportions (Table 5), colour in preservative, and patterning.

Hind limbs long (TL/SVL = 0.62 ± 0.03, range 0.56–0.70). Head approximately as long as broad (HL/HW = 0.92±0.06, range 0.77–1.06; eye diameter greater than eye to naris distance (ED/EN = 1.53±0.19, range 1.08–1.98), and eye to naris distance versus internarial span variable (EN/IND = 0.8 ± 0.1, range 0.62–1.16). Ventral surface is smooth. Brown glandular nuptial pad on first and second finger and inner palmar tubercle in breeding males.

Colour in life. Dorsal surfaces rich copper to tan or dark brown or with green tinge, sometimes with burnt

orange wash towards groin; a posteriorly oriented inter-orbital bar that extends as a mid-dorsal stripe, finely edged in black and highly variable in length and shape with uneven edges, often not continuous and usually terminating on the upper back but occasional extending to the mid-sacral region; remainder of dorsal surface either sparsely flecked with small black spots or a few larger blotches or a combination of both (Fig. 8). A dark triangular patch on either side of the snout with the apex at the external nostril but does not include the immediate area around the nostril. A dark lateral head-stripe extends from slightly posterior of the nostril, through eye, curves over tympanum, and extends posteriorly down anterior margin of the tympanum, sometimes including upper third of the tympanum, terminating above axilla; width variable but in all cases posterior margin is widest; bordered above and below by fine cream or fawn line that is sharply demarcated against the dark stripe. Loreal region is light coloured with darker patches along the upper jaw (maxilla).

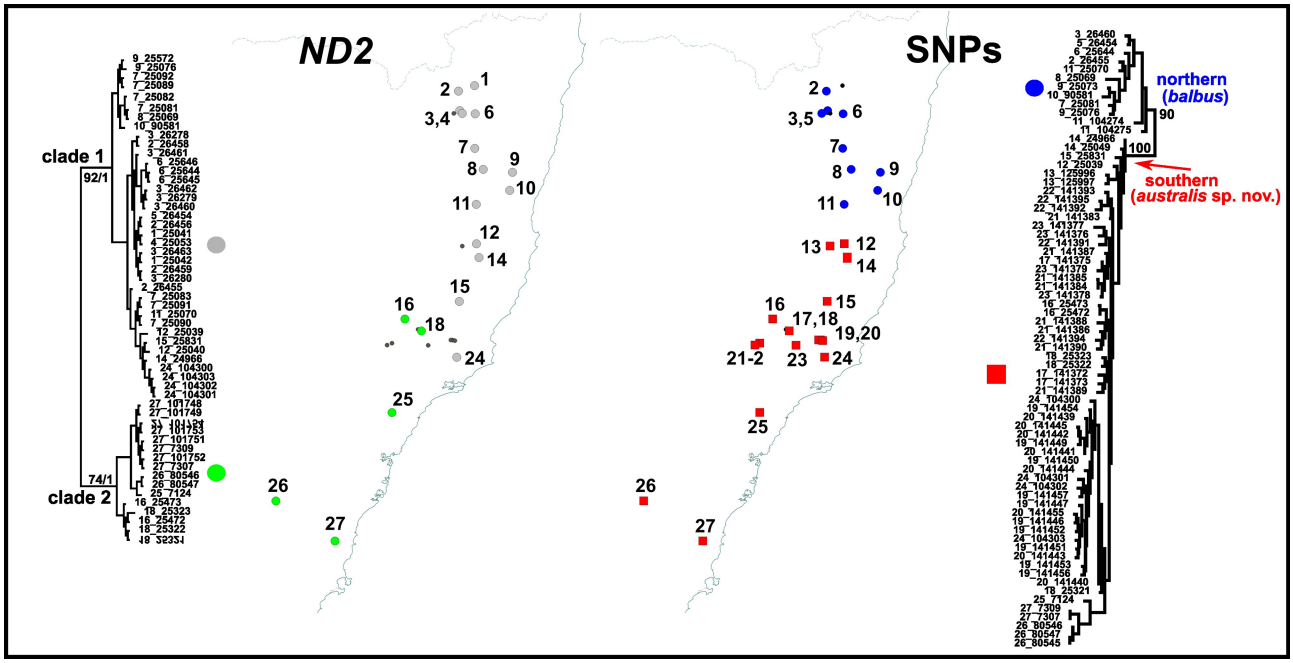

FIGURE 10. Comparison of geographic pattern of relationships among mitochondrial DNA sequences and nuclear genetic (SNP) groups, highlighting the discordance in relationships among samples inferred from the two genomic compartments. Refer to Table 1 for locality code information and ABTC numbers.

Lateral surface and groin flushed with a reddish tan or peach, without spots or blotches, these surfaces noticeably lighter than dorsum, with colour diffusing gradually into the ventral colouration. Venter including throat and ventral surface of thighs immaculate white, cream or with a light lemon-yellow wash; lower jaw edged with diffuse brown wash.

In adults, iris dark brown merging into golden brown above pupil with an iridescent pale blue dorsal crescent.

Upper surfaces of the legs, feet, arms and hands with dark transverse cross bars, not extending on to posterior thighs; posterior surface of thighs uniform brown to burnt orange brown and slightly mottled; unpigmented triangular area with distinct closely grouped tubercles beneath the cloaca; anterior lateral edge of shank, posterior lateral edges of arms, and plantar surface of feet and tarsus dark brown; lateral palmar surfaces of outer fingers dark.

Reproductive call. The male advertisement call comprises one to four short notes repeated in rapid succession. Because of the variation in the number of notes in the call we provide means and ranges for measurements of call traits for the first two notes. Mean duration for a two note call is 1.34 s (range 1.29–1.35 s). There are commonly two notes in the call with the first of longer duration than the second (mean 0.69 and 0.45 s, ranges 0.30 0.70, and 0.18–0.45 respectively), with a short inter-note interval (mean 0.36 s), and with more pulses in the first note than the second (mean 8.6, and 6.2, ranges 5–11, 3–8 respectively). Pulse duration is much shorter (mean 0.015 s, range 0.011–0.017) than inter-pulse interval (0.083 s, range 0.080–0.088). Pulse structure varies within notes, with single pulses produced initially, followed by a series of pulses produced in couplets (Table 2, Fig. 9). The second note has more couplets than the first note (Table 2, Fig. 9). Pulse repetition rate is lower in the first note than the second (15.95 and 20.86 respectively) (Table 2). Mean inter-call interval is 4 s (range 3–5 s) and males usually produce

a series of calls before a period in which all calling ceases. Calling occurs during the spring and summer seasons when climatic conditions are suitable (MM pers. obs. Details of suitable conditions not reported here). Within the calling season, calling is initiated by a male, and this stimulates calling in nearby males and a chorus of calls with a short lag time or small overlap between the call of one male and a nearby male occurs. The male encounter call is a single note of moderate duration (mean 0.23 s, range 0.24–0.48 s) composed of dense pulses and is often uttered among advertisement calls and males may produce several encounter calls in succession (Fig. 9C). The dominant frequency of the notes in the advertisement call differs slightly with the first note having a higher frequency than the second (732 and 742 Hz, range 650 to 768Hz). The DF of the encounter call (704 Hz, range 632 to 831Hz) is slightly lower than the advertisement call. Advertisement and encounter calls are not frequency modulated. Call recordings have been deposited at the Australian Museum as a multimedia record attached to the database record for the holotype R.188750.

Distribution. *Mixophyes australis* **sp. nov.** is distributed from the Carrai Plateau on the southern side of the Macleay River in New South Wales south to the Cann River catchment in East Gippsland Victoria (Fig. 1). All records are from wet forest habitats in drainages that flow to the east of the Great Dividing Range. Its distribution includes three bioregions (Thackway & Cresswell 1995), the southern quarter of the North East Coast, the Sydney Basin and Southeast Corner bioregions. This species occurs across almost all available elevations in these bioregions. For example, in the Barrington Range it occurs from 390 m asl (Sharpes Creek) up to 1230 m asl (Dilgry River), and in the Sydney Basin from 50 m asl (Stanwell Tops) up to 1000 m asl (Blackheath, Mount Irvine and Mount Wilson). In the Southeast Corner bioregion, the highest altitude record is 810 m asl (Bombala), and the southernmost location, at Chandlers Creek, East Gippsland, is at 280 m asl.

We note that genetic analysis of southern NSW and north-eastern Victorian populations of *Mixophyes* from spirit preserved vouchers should be considered. This region has a little appreciated biogeographic barrier immediately to the north of Nowra, which in some recent studies on frogs is manifested as a deep species level break that also may have an influence as a barrier to north–south movement more widely on the coastal fauna of southern coastal NSW and north-eastern Victoria (Mahony *et al.* 2020, 2021).

Habitat and Ecology. The ecological requirements of adults and larvae of *M. australis* **sp. nov.** are reasonably well known. This species is found in association with first and second order permanent and ephemeral streams in temperate and sub-tropical rainforest, wet sclerophyll forest, and also in moist gullies in dry forest (Gillespie & Hines 1999). Statistical modelling using presence and absence data and 24 environmental predictors from the northern portion of the range of *M. australis* **sp. nov.** (Barrington and Hastings Ranges) showed a preference for the interiors of large forest tracts in areas with relatively cool mean annual temperatures, at sites that were typically free from any disturbance with a thick canopy and relatively simple understorey (NSW NEFBS 1994).

Adult males spend most of their lives in the riparian zone where they shelter under leaf litter or burrow into the topsoil or under debris such as logs when inactive during all seasons. Males leave these protected sites during active periods of calling from spring through to autumn, at which times they call from stream banks or among rocks next to the riffle zone where oviposition occurs (Knowles *et al.* 2015). Females move away from the riparian zone and, like males, shelter under leaf litter and burrow into the topsoil during periods of inactivity (Mahony pers. obs.). Larvae are nektonic and occupy pools between riffle zones in small streams (Daly 1998).

Eggs and larvae. Embryonic development and larval morphology were described by Anstis (2013), from specimens collected at five locations that include three within the range of *M. australis* **sp. nov.** (Tirrill Creek, Bulga State Forest; Sharpes Creek, Barrington Range; and Gap Creek, Central Coast Range), and two within the range of *M. balbus* (near Point Lookout, New England Range, near type locality; Coombadjha Creek, Gibraltar Range). No morphological features that distinguished among these samples were reported (Anstis 2013), but it is possible that variation was present that was not reported since Anstis was not aware of the presence of two species amongst her samples. *Mixophyes australis* **sp. nov.** deposit eggs among small pebbles or debris in gently flowing shallow water (Knowles *et al.* 2015).

Etymology. The specific Latin epithet *australis* refers to the species being the most southerly distributed member of *Mixophyes*.

Assessment of IUCN threat category for *M. australis* sp. nov. We assess *M. australis* **sp. nov.** to be Endangered under category A2(a)B2(a)(b). There is an estimated population reduction of >30% over the past decade and the causes may not have ceased (Supplementary Text. Figs S4—S6). The Area of Occupancy (AOO) in the decade 2011-2020 is 188 km^2, which is well below the threshold level of 2,000 km^2, and there is evidence of a continuing

population reduction based on mapping of database records over three generations (Supplementary Text Fig. S1). *Mixophyes australis* **sp. nov.** is known over a relatively large range (EOO for all records is a convex hull of 81,578 km^2, and an alpha-hull of 2,734 km^2). In the decade from 2001-2010, these values reduced to 33,003 km^2 and 1,495 km^2 respectively, which demonstrates the rate of contraction of the distribution. Mapping of the records reveals declines and disappearances of populations in the southern two thirds of the distribution (Supplementary Text Fig. S1).

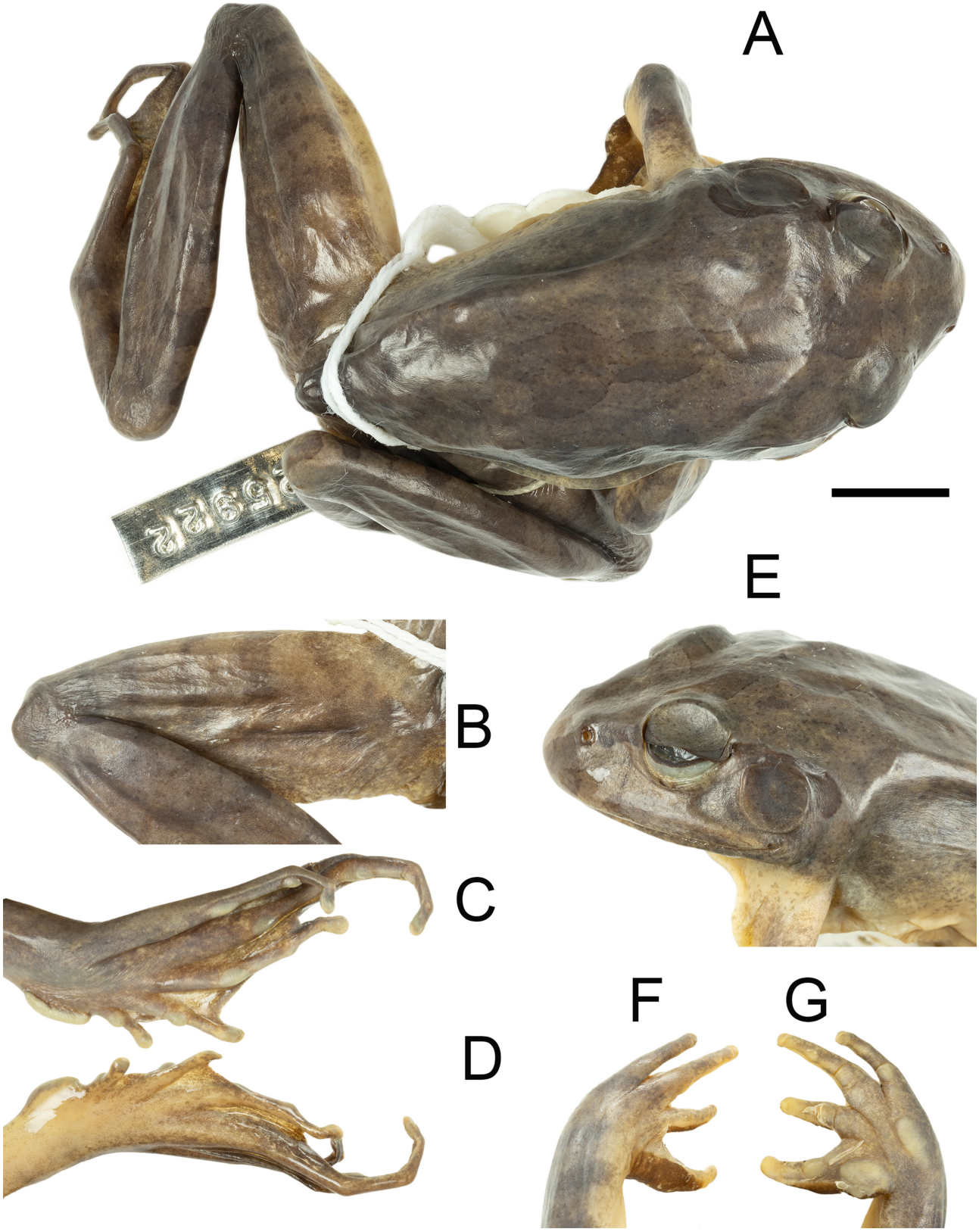

FIGURE 11. Images of *Mixophyes balbus* holotype, scale bar 10 mm (AMS R25922).

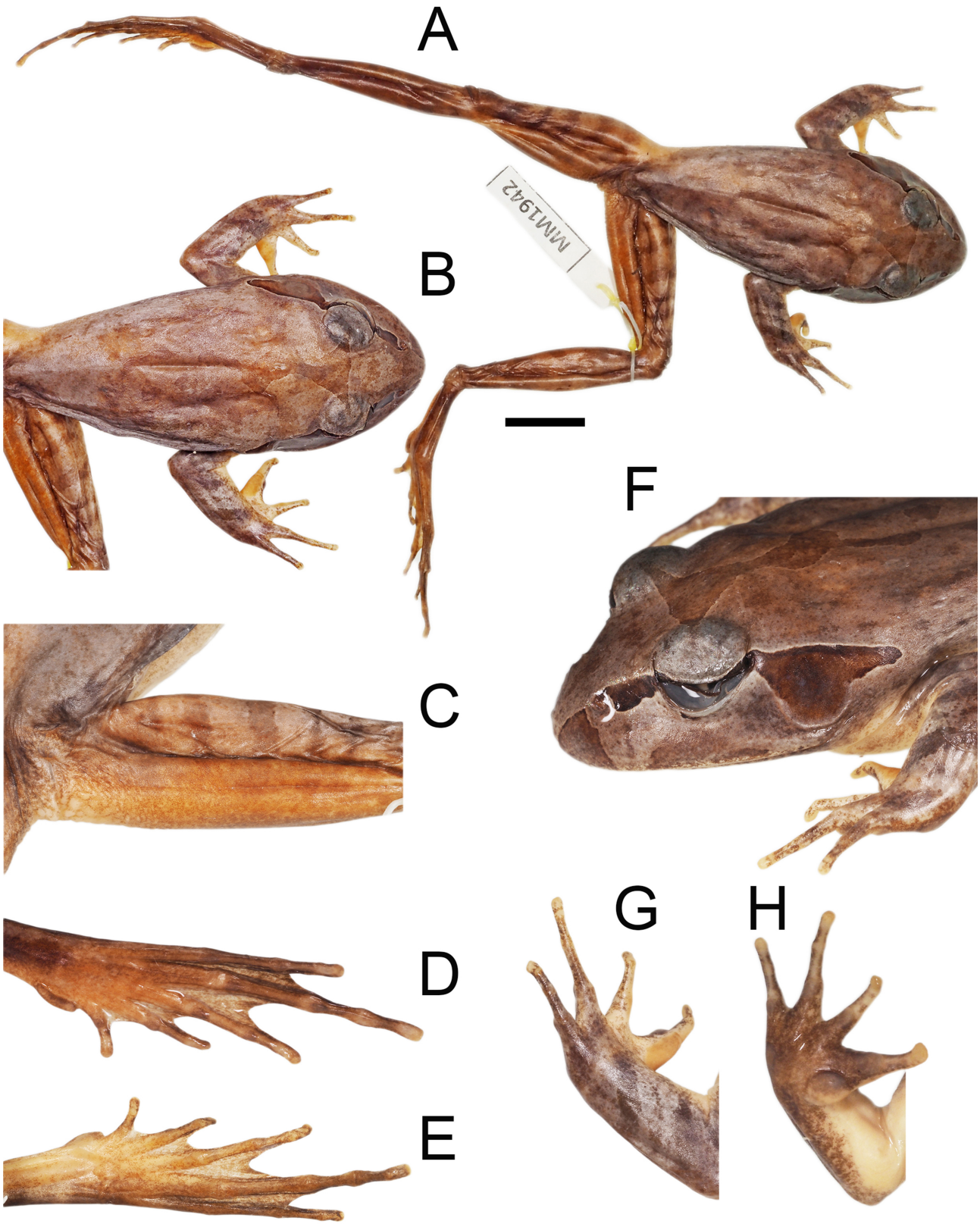

FIGURE 12. Images of *Mixophyes australis* **sp. nov**. holotype, scale bar 10 mm (AMS R188750).

Gillespie *et al.* (2014) reviewed the conservation status of the Victorian and southern NSW populations and noted that prior to 1983 only five specimens had been recorded in Victoria from three localities. To our knowledge there have been no observations of this species in Victoria in the past two decades. Field surveys in the south-eastern forest region and the Greater Blue Mountains area of NSW conducted in the past decade have documented the

loss of populations in this large region (Daly & Craven 2011, Mahony unpubl. data). Fortunately, this decline has occurred only in the southern two thirds of the range and intensive surveys in the northern third including the Central Coast, Myall, Barrington and Hasting Ranges show that the species remains in these locations (this study, see Supplementary Text) and that populations have remained stable over the past decade (Mahony 2007, 2013).

Because genetic evidence indicates that there are two subpopulations of *M. australis* **sp. nov.** that are separated by the Hunter River valley, a northern subpopulation equivalent to genetic clusters S1, S2 and S3, and a southern subpopulation equivalent to cluster S4, we also assess their threat status (Supplementary Text). The northern subpopulation (i.e., Myall Range, Barrington Range and Hastings Range), and the southern subpopulation (Central Coast Range, Blue Mountains and Illawarra region to east Gippsland) meet the conservation status assessment criteria for Endangered 2B1a,b (Supplementary Text Table S1).

Threats considered to impact *M. australis* **sp. nov**. are the same as those for *M. balbus* but the extensive contraction of the species' distribution in the southern portion of its former range is a matter of considerable conservation concern. The recovery of many populations in the northern third of its range provides an option for the return of this frog to the southern part of its former distribution. In support of such an action, successful captive husbandry of *Mixophyes* has been reported (Banks *et al.* 2014) and protocols for translocations and reintroduction for Bd affected species are available (Scheele *et al.* 2021). While the northern populations may provide frogs for reintroduction, it should be noted that northern and southern populations are genetically very divergent, and the northern populations occupy an environmental envelope where the organism x environment interaction could be more favourable to the frog than may occur in the southern part of the range. Given the above, a precautionary approach using an experimental evaluation of the survival of northern frogs in an environment that they have not experienced in their recent evolutionary history including a recent period of their exposure to Bd, may be an initial action.

Acknowledgments

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