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Systematic assessment of the brown tree frog (Anura: Pelodryadidae: *Litoria ewingii*) reveals two endemic species in South Australia

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Abstract

The brown tree frog (*Litoria ewingii*) is a relatively widespread, commonly encountered pelodryadid frog from south-eastern Australia, known for its characteristic whistling call. The distribution of *Litoria ewingii* spans over more than 350,000 km^2, encompassing a range of moist temperate habitats, and is fragmented by well-known biogeographic barriers. A preliminary analysis of mitochondrial DNA sequences revealed evidence for deep phylogenetic structure between some of these fragmented populations. In this study, we sought to re-evaluate the systematics and taxonomy of *Litoria ewingii sensu lato* by analysing variation in nuclear and mitochondrial DNA, adult morphology and male advertisement calls throughout the species' range. Our analyses reveal two additional, deeply divergent and allopatric lineages in South Australia. We herein re-describe *Litoria ewingii* from Tasmania, southern New South Wales, Victoria and south-eastern South Australia, resurrect the name *Litoria calliscelis* for a species occurring in the Mount Lofty Ranges and Fleurieu Peninsula in South Australia, and describe a new species, *Litoria sibilus* **sp. nov.**, endemic to Kangaroo Island.

Key words: mtDNA, single-nucleotide polymorphisms, biogeography, Kangaroo Island, Murray River Basin

Introduction

The *Litoria ewingii* Group (*sensu* Tyler & Davies 1978) comprises seven small to medium sized pelodryadid frog species distributed throughout moist temperate regions of southern and eastern Australia, with the range of one species also including isolated areas of the tropical northeast. Members of this species group include *Litoria ewingii* (Duméril & Bibron, 1841), *Litoria jervisiensis* (Duméril & Bibron, 1841), *Litoria littlejohni* (White, Whitford & Mahony, 1994), *Litoria paraewingi* Watson, Loftus-Hills & Littlejohn 1971, *Litoria revelata* Ingram, Corben & Hosmer, 1982, *Litoria verreauxii* (Duméril, 1853) and *Litoria watsoni* Mahony, Moses, Mahony, Lemckert & Donnellan, 2020. Species in this group are known for their whistle-like male advertisement calls, which comprise a series of repeated, high-frequency pulsed notes.

The *Litoria ewingii* Group has been studied extensively as a model for amphibian hybridisation, with a particular focus on the role of pre- and post-mating reproductive isolation mechanisms in maintaining species boundaries at hybrid zones (Dennington 1990, Gartside 1972, Littlejohn 1965, Littlejohn 1976, Littlejohn & Watson

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1983, Melville *et al.* 2017, Smith *et al.* 2012, Smith *et al.* 2013a, Smith *et al.* 2013b, Watson *et al.* 1971, Watson 1972, Watson & Littlejohn 1978, Watson *et al.* 1985). Much of this research has focused on the dynamics of a narrow hybrid zone between *Litoria ewingii* and *L. paraewingi* in south-central Victoria. Despite a near absence of pre-mating isolating factors between the species (i.e., a lack of obvious differences in mating call structure or morphology that might prevent effective mating), the species boundaries are maintained by a high degree of post-mating genetic incompatibility. Artificial hybridisation experiments have demonstrated that crosses between male *Litoria paraewingi* and female *L. ewingii* result in 67–100% of progeny developing lethal anopthalmia, a disorder characterised by abnormal development of the cephalic region, typically expressed in embryos by a lack of eyes (Watson *et al.* 1971, Watson 1972). The hybrid zone between these species has been termed a "tension" zone (Smith *et al*. 2013b) and appears to have remained relatively stable over the past 50 years, shifting southward by one kilometre during that period (Smith *et al.* 2013a).

Although the interactions between members of the *Litoria ewingii* Group have been comprehensively studied, our understanding of the phylogenetic relationships, systematics and taxonomy of the group remains relatively unsettled. This is due largely to their morphological and acoustically conserved nature, as highlighted by the recent description of the morphologically cryptic *Litoria watsoni* (Mahony *et al.* 2020). The identification and classification of cryptic diversity is of high research priority given it has significant implications for effective conservation, both in providing a metric for biodiversity and allowing for scientific and legal recognition of taxonomic units for conservation management (Coates *et al.* 2018, Dissanayake *et al.* 2022).

Litoria ewingii fits the criteria of a species likely to include cryptic diversity given its widespread distribution, limited dispersal capability and the geographic disjunction of some populations throughout its range (Donnellan *et al*. 1993). We aimed to clarify the systematics and taxonomy of *Litoria ewingii sensu lato* by examining variation in mitochondrial and nuclear DNA, adult morphology and male advertisement calls throughout the species' range.

Materials and methods

To make this paper easier for the reader to follow, we use the final specific epithets of *Litoria ewingii* (Duméril & Bibron, 1841), *Litoria calliscelis* (Peters, 1874) and *Litoria sibilus* **sp. nov.** throughout the manuscript rather than using an initial group nomenclature that we would change in the taxonomy section.

Mitochondrial DNA extraction and analysis. Nucleotide sequences of the mitochondrial *NADH subunit 4* (*ND4*) gene were obtained from *Litoria ewingii* (n=46), *L. calliscelis* (n=4), *L. sibilus* (n=7), and all other members of the eastern *L. ewingii* group (*L. jervisiensis*, *L. revelata*, *L. littlejohni*, *L. watsoni*, *L. paraewingi* and *L. verreauxii*) for molecular genetic analyses (Table 1). Total genomic DNA was extracted from ethanol-preserved tissues (liver, muscle, toe tip) using a DNeasy® Blood and Tissue Kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's protocols for purification of genomic DNA from animal tissues. A fragment of the *ND4* gene was PCR amplified and directly sequenced using the primers: 5'-TGA CTA CCA AAA GCT CAT GTA GAA GC-3' (*ND4*) and 5'-GGT YAC GAG YAA TTA GCA GTT CT-3' (*Limno2*). PCRs were carried out in 25 µL reactions consisting of 2.5 µL of template DNA, 5 µL of MyTaq™ Red Reaction Buffer, 0.25 µL of each primer, 0.2 µL of Bioline MyTaqTM Red DNA Polymerase and 16.8 µL of autoclaved water. Thermocycling was performed on an Eppendorf Mastercycler EpS (Eppendorf, Hamburg, Germany) under the following cycling protocol: (1) initial denaturation at 94ºC for 3 min, (2) 10 cycles involving a denaturation step of 94ºC for 45 seconds, annealing at 60ºC for 1 min and extension at 72ºC for 1 min, with the annealing temperature decreased by 1ºC per cycle, (3) 34 cycles of 94ºC for 45 sec, 50ºC for 1 min and 72ºC for 1 min, (4) final extension step of 72ºC for 6 min with samples kept at a holding temperature of 11ºC. Amplification products were visualised on 1.5% agarose gels, purified using ExoSap-ITTM (USB Corporation, Cleveland, Ohio, USA) and sequenced in both directions at Macrogen (Seoul, South Korea). Sequence chromatograms were edited and checked for quality using Geneious Prime v. 2022.1.1. Sequences were deposited in GenBank (accession numbers: OR545978–546045, 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 1000 standard bootstrap pseudo-replicates (Hoang *et al.* 2017).

TABLE 1. *Litoria ewingii* Group specimens examined for molecular genetic analyses. Institution codes: ABTC—Australian Biological Tissue Collection, South Australian Museum; NMV—Museums Victoria; AMS—Australian Museum; SAMA—South Australian Museum; QM—Queensland Museum; ANWC—Australian National Wildlife Collection; TMAG—Tasmanian Museum and Art Gallery. State abbreviations: SA—South Australia; Vic—Victoria; NSW—New South Wales; Tas—Tasmania; Qld—Queensland; ACT—Australian Capital Territory. *ND4* column refers to GenBank accession numbers.

Tissue RegNum	Species	ND4	SNP	Locality	State	Latitude	Longitude	Voucher institution	Voucher RegNum
ABTC14683	*Litoria calliscelis*	OR546026	y	Highbury	SA	-34.830	138.680	SAMA	R34901
ABTC33268	*Litoria calliscelis*	OR546027	-	22 km ESE Mt Compass	SA	-35.428	138.809	SAMA	R49211
ABTC74464	*Litoria calliscelis*	OR546028	-	1.5 km NE Norton Summit	SA	-34.912	138.738	SAMA	R54982
ABTC93424	*Litoria calliscelis*	-	y	1.8 km NNE Caloote	SA	-34.951	139.272	SAMA	R59158
ABTC93463	*Litoria calliscelis*	-	y	4.6 km ENE Pellaring Flat	SA	-34.859	139.442	SAMA	R59174
ABTC94832	*Litoria calliscelis*	OR546029	y	0.7 km NE Pijanbilli Lodge	SA	-35.546	138.374	SAMA	R53569
ABTC94968	*Litoria calliscelis*	-	y	Wangoola Homestead	SA	-35.566	138.367	SAMA	R53645
ABTC12434	*Litoria ewingii*	OR546010	y	3 km E Toora	Vic	-38.670	146.370	SAMA	R39003
ABTC12435	*Litoria ewingii*	OR546012	-	3 km E Toora	Vic	-38.670	146.370	SAMA	R39004
ABTC12436	*Litoria ewingii*	MT497817	-	3 km E Toora	Vic	-38.670	146.370	SAMA	R39005
ABTC12437	*Litoria ewingii*	OR546013	-	3 km E Toora	Vic	-38.670	146.370	SAMA	R39006
ABTC132072	*Litoria ewingii*	OR546001	y	Halls Gap	Vic	-37.173	142.545	SAMA	R61927
ABTC132074	*Litoria ewingii*	-	y	15km E Mt Gambier	Vic	-37.814	141.001	SAMA	R61929
ABTC132075	*Litoria ewingii*	OR546004	y	15 km E Mt Gambier	Vic	-37.814	141.001	SAMA	R61930
ABTC142838	*Litoria ewingii*	-	y	Ferndale, Strzelecki Ranges	Vic	-38.310	145.971	SAMA	R62478
ABTC14721	*Litoria ewingii*	OR545997	y	Penola State Forest	SA	-37.470	140.830	SAMA	R34908
ABTC14736	*Litoria ewingii*	OR545993	y	Penola	SA	-37.380	140.830	SAMA	R34913
ABTC16984	*Litoria ewingii*	OR545999	y	near Bessie Belle	Vic	-38.170	141.970	SAMA	R35813
ABTC17602	*Litoria ewingii*	OR546015	y	Boydtown	NSW	-37.100	149.880	SAMA	R42612
ABTC17605	*Litoria ewingii*	OR546014	y	4 km S Narrabarba	NSW	-37.270	149.800	SAMA	R42615
ABTC23116	*Litoria ewingii*	OR546020	-	Pigsty Ponds	Tas	-43.487	146.727	TMAG	C872
ABTC37513	*Litoria ewingii*	-	y	17.3 km WNW Kongorong Forest Reserve	SA	-37.902	140.393	SAMA	R49566
ABTC37521	*Litoria ewingii*	OR545994	-	0.5 km E Carpenters Rocks	SA	-37.911	140.400	SAMA	R49578
ABTC37533	*Litoria ewingii*	MT497818	y	20 km WNW Millicent Airport	SA	-37.507	140.149	SAMA	R49588
ABTC58288	*Litoria ewingii*	OR545995	y	4.3 km E Dunmore Homestead	SA	-36.236	140.068	SAMA	R45982

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TABLE 1. (Continued)

Tissue RegNum	Species	ND4	SNP	Locality	State	Latitude	Longitude	Voucher institution	Voucher RegNum
ABTC58652	*Litoria ewingii*	OR546002	y	Tarpeena	SA	-37.699	140.798	SAMA	R49330
ABTC70043	*Litoria ewingii*	OR545996	y	9 km SW Millicent	SA	-37.633	140.275	SAMA	R53776
ABTC70616	*Litoria ewingii*	y	y	4.6 km SSW Bald Hill	SA	-36.553	140.004	SAMA	R53370
ABTC7237	*Litoria ewingii*	OR545992	-	Lucindale	SA	-36.970	140.370		
ABTC94804	*Litoria ewingii*	OR545998	y	7.7 km SE Kangaroo Hill	SA	-37.206	139.928	SAMA	R53767
EBU87903	*Litoria ewingii*	OR546007	y	Jancourt Nature Conservation Reserve	Vic	-38.391	143.191		
JJLR5379	*Litoria ewingii*	OR546023	y	Gladstone	Tas	-40.936	148.087	TMAG	C1527
JJLR5380	*Litoria ewingii*	-	y	Gladstone	Tas	-40.936	148.087	TMAG	C1528
JJLR5381	*Litoria ewingii*	OR546022	y	Pioneer	Tas	-41.071	147.925	TMAG	C1529
JJLR5383	*Litoria ewingii*	OR546024	y	Penguin	Tas	-41.140	146.062	TMAG	C1530
JJLR5389	*Litoria ewingii*	OR546025	y	E of Temma	Tas	-41.215	144.902	TMAG	C1531
JJLR5393	*Litoria ewingii*	-	y	Zeehan	Tas	-41.819	145.125	TMAG	C1532
JJLR5394	*Litoria ewingii*	-	y	Zeehan	Tas	-41.819	145.167	TMAG	C1533
JJLR5396	*Litoria ewingii*	OR546021	y	~15 km W Eaglehawk Neck Tasman Peninsula	Tas	-43.038	147.747	TMAG	C1534
JJLR5398	*Litoria ewingii*	-	y	Bicheno	Tas	-41.930	148.228	TMAG	C1535
JJLR5894	*Litoria ewingii*	OR546016	y	Colquhoun/Boyanga Gidi State Forest	Vic	-37.743	148.044	NMV	D 76455
JJLR5895	*Litoria ewingii*	y	y	Colquhoun/Boyanga Gidi State Forest	Vic	-37.743	148.044	NMV	D 76456
JJLR5901	*Litoria ewingii*	OR546011	y	Bunyip State Forest	Vic	-37.957	145.915	NMV	D 76457
JJLR5902	*Litoria ewingii*	OR546018	y	Bunjil Bushland Reserve	Vic	-37.647	145.246	NMV	D 76458
JJLR5908	*Litoria ewingii*	OR546006	y	Ross Creek State Forest	Vic	-37.668	143.697	NMV	D 76459
JJLR5909	*Litoria ewingii*	OR546003	y	Mount Clay State Forest	Vic	-38.226	141.686	NMV	D 76460
JJLR5910	*Litoria ewingii*	OR546000	y	Mount Clay State Forest	Vic	-38.192	141.680	NMV	D 76461
JJLR5913	*Litoria ewingii*	OR546005	y	Halls Gap	Vic	-37.171	142.542	NMV	D 76462
NMVZ11155	*Litoria ewingii*	-	y	Ming Ming Swamp, Grampians	Vic	-37.331	142.193	NMV	D 75485
NMVZ12831	*Litoria ewingii*	OR546019	y	Mount Disappointment	Vic	-37.377	145.096		
NMVZ12832	*Litoria ewingii*	-	y	Heathcote Junction	Vic	-37.377	145.096		
NMVZ12833	*Litoria ewingii*	y	y	Mount Disappointment	Vic	-37.377	145.096		

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TABLE 1. (Continued)

Tissue RegNum	Species	ND4	SNP	Locality	State	Latitude	Longitude	Voucher institution	Voucher RegNum
NMVZ12834	*Litoria ewingii*	-	y	Heathcote Junction	Vic	-37.377	145.096		
NMVZ12835	*Litoria ewingii*	-	y	Heathcote Junction	Vic	-37.377	145.096		
NMVZ13203	*Litoria ewingii*	KC183742	-	Castella	Vic	-37.517	145.420		
NMVZ13204	*Litoria ewingii*	KC183741	-	Castella	Vic	-37.517	145.420		
NMVZ13205	*Litoria ewingii*	-	y	Pakenham	Vic	-38.032	145.433		
NMVZ13206	*Litoria ewingii*	-	y	Pakenham	Vic	-38.032	145.433		
NMVZ13208	*Litoria ewingii*	-	y	Pakenham	Vic	-38.032	145.433		
NMVZ13222	*Litoria ewingii*	KC183740	-	Castella	Vic	-37.517	145.420		
NMVZ13223	*Litoria ewingii*	KC183733	-	Castella	Vic	-37.517	145.420		
NMVZ13375	*Litoria ewingii*	KC183735	-	Yarra Valley	Vic	-37.562	145.417		
NMVZ27038	*Litoria ewingii*	-	y	Castella	Vic	-37.504	145.405		
NMVZ34908	*Litoria ewingii*	-	y	Dowd Morass Wildlife Reserve	Vic	-38.145	147.146		
NMVZ60370	*Litoria ewingii*	-	y	Wombat Creek	Vic	-37.771	148.243	NMV	D 77301
NMVZ60376	*Litoria ewingii*	-	y	Wombat Creek	Vic	-37.772	148.245	NMV	D 77306
NMVZ60387	*Litoria ewingii*	-	y	Wombat Creek	Vic	-37.771	148.243	NMV	D 77315
NMVZ60403	*Litoria ewingii*	-	y	Providence Ponds Nature Conservation Reserve	Vic	-37.934	147.294	NMV	D 77809
NMVZ60617	*Litoria ewingii*	OR546008	-	Anglesea Heath	Vic	-38.349	144.136	NMV	D 77211
NMVZ60627	*Litoria ewingii*	-	y	Anglesea Heath	Vic	-38.402	144.145	NMV	D 77223
NMVZ60629	*Litoria ewingii*	OR546009	y	Anglesea	Vic	-38.402	144.145	NMV	D 77221
NMVZ60630	*Litoria ewingii*	y	y	Anglesea	Vic	-38.402	144.145	NMV	D 77220
NMVZ60633	*Litoria ewingii*	-	y	Anglesea Heath	Vic	-38.402	144.145	NMV	D 77218
ABTC13646	*Litoria ewingii* ("northern")	y	y	Blowering	NSW	-35.470	148.280		
ABTC13700	*Litoria ewingii* ("northern")	OR546041	y	Tumbarumba	NSW	-35.850	148.120		
AMSR140834	*Litoria ewingii* ("northern")	-	y	Bogong Creek, Kosciusko National Park	NSW	-36.250	148.250	AMS	AMSR140834

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TABLE 1. (Continued)

Tissue RegNum	Species	ND4	SNP	Locality	State	Latitude	Longitude	Voucher institution	Voucher RegNum
AMSR188372	*Litoria ewingii* (“northern”)	-	y	Tumbarumba	NSW	-35.783	147.986	AMS	AMSR188372
AMSR188373	*Litoria ewingii* (“northern”)	-	y	Tumbarumba	NSW	-35.783	147.986	AMS	AMSR188373
ABTC25157	*Litoria jervisiensis*	y	y	Awabakal Nature Reserve, Dudley	NSW	-33.000	151.720		
ABTC25158	*Litoria jervisiensis*	y	y	Awabakal Nature Reserve, Dudley	NSW	-33.000	151.720		
ABTC25451	*Litoria jervisiensis*	MT497819	y	Darkes Forest	NSW	-34.220	150.900		
ABTC25839	*Litoria jervisiensis*	MT497820	y	Mungo Brush, Myall Lakes National Park	NSW	-32.500	152.300		
ABTC80824	*Litoria jervisiensis*	y	y	Kioloa	NSW	-35.550	150.370		
ABTC90506	*Litoria jervisiensis*	y	y	Kurnell Peninsula	NSW	-34.026	151.218		
ABTC145264	*Litoria littlejohni*	MT497842	-	Kings Tableland	NSW	-33.814	150.438		
ABTC150907	*Litoria littlejohni*	MT497840	-	Andersons Fire Trail, Kings Plateau	NSW	-33.803	150.426		
ABTC150908	*Litoria littlejohni*	MT497841	-	Andersons Fire Trail, Kings Plateau	NSW	-33.803	150.426		
ABTC97410	*Litoria littlejohni*	MT497833	-	Olney State Forest	NSW	-33.098	151.350		
ABTC97411	*Litoria littlejohni*	MT497831	-	Olney State Forest	NSW	-33.067	151.339		
ABTC12855	*Litoria paraewingi*	MT497821	y	7 km N Merton	Vic	-36.930	145.750	SAMA	R44074
ABTC12856	*Litoria paraewingi*	MT497822	y	6 km W Strathbogie	Vic	-36.880	145.680	SAMA	R44066
ABTC17553	*Litoria paraewingi*	-	y	East Albury	NSW	-36.080	146.930	SAMA	R42148
ABTC17554	*Litoria paraewingi*	-	y	East Albury	NSW	-36.080	146.930	SAMA	R42149
ABTC40923	*Litoria paraewingi*	MT497823	-	Reef Hills State Park	Vic	-36.600	145.950	SAMA	R34675
NMVZ13521	*Litoria paraewingi*	-	y	Murrindindi	Vic	-37.383	145.482		
NMVZ13541	*Litoria paraewingi*	-	y	Glenburn	Vic	-37.432	145.459		
NMVZ13608	*Litoria paraewingi*	OR546040	y	Beechworth	Vic	-36.358	146.700		
NMVZ26990	*Litoria paraewingi*	-	y	Marysville	Vic	-37.492	145.748		
ABTC25078	*Litoria revelata*	OR545978	-	Wild Cattle Creek State Forest	NSW				
ABTC25996	*Litoria revelata*	OR545979	-	Coolongolook State Forest	NSW	-32.217	152.317		
ABTC26010	*Litoria revelata*	OR545980	-	Pine Creek State Forest	NSW				
ABTC80826	*Litoria revelata*	OR545981	-	Maxwell Flat	NSW	-31.480	152.180	SAMA	R68987

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TABLE 1. (Continued)

Tissue RegNum	Species	ND4	SNP	Locality	State	Latitude	Longitude	Voucher institution	Voucher RegNum
ABTC86373	*Litoria revelata*	OR545982	-	Nightcap Range, Whian Whian State Forest	NSW	-28.600	153.377	ANWC	A01997
ABTC102390	*Litoria revelata* (north Qld)	MT497815	-	Zillie Falls Road, Ellinjaa	Qld	-17.467	145.649	QM	J90806
ABTC102391	*Litoria revelata* (north Qld)	MT497816	-	Zillie Falls Road, Ellinjaa	Qld	-17.467	145.649		
ABTC80815	*Litoria revelata* (north Qld)	OR545983	-	Millaa Millaa	Qld	-17.490	145.610		
ABTC80816	*Litoria revelata* (north Qld)	OR545984	-	Millaa Millaa	Qld	-17.490	145.610		
ABTC33479	*Litoria sibilus* **sp. nov.**	OR545990	y	1.5 km NW Rocky River, Flinders Chase National Park	SA	-35.940	136.729	SAMA	R37403
ABTC33511	*Litoria sibilus* **sp. nov.**	OR545991	y	Larrikin Lagoon, Flinders Chase National Park	SA	-35.835	136.687	SAMA	R37476
ABTC7188	*Litoria sibilus* **sp. nov.**	OR545985	y	South West River, Kangaroo Island	SA	-35.980	136.870		
ABTC7189	*Litoria sibilus* **sp. nov.**	OR545986	y	South West River, Kangaroo Island	SA	-35.980	136.870	SAMA	R20183
ABTC7190	*Litoria sibilus* **sp. nov.**	OR545989	y	South West River, Kangaroo Island	SA	-35.980	136.870	SAMA	R20184
ABTC7192	*Litoria sibilus* **sp. nov.**	OR545987	y	South West River, Kangaroo Island	SA	-35.980	136.870	SAMA	R20186
ABTC7193	*Litoria sibilus* **sp. nov.**	OR545988	y	South West River, Kangaroo Island	SA	-35.980	136.870	SAMA	R20187
ABTC113113	*Litoria verreauxii*	y	-	Bairnsdale	Vic	-37.882	147.576		
ABTC113122	*Litoria verreauxii*	y	y	Macleods Morass	Vic	-37.837	147.623		
ABTC113125	*Litoria verreauxii*	-	y	Reedy Creek	Vic	-37.057	147.327		
ABTC12630	*Litoria verreauxii*	MT497828	y	Camden	NSW	-34.050	150.670	SAMA	R40839
ABTC12644	*Litoria verreauxii*	OR546030	y	Paddy's River crossing, Penrose State Forest	NSW	-34.650	150.130	SAMA	R40862
ABTC13639	*Litoria verreauxii*	OR546035	y	Tantangara Dam	NSW	-35.800	148.670		

...Continued on the next page

TABLE 1. (Continued)

Tissue RegNum	Species	ND4	SNP	Locality	State	Latitude	Longitude	Voucher institution	Voucher RegNum
ABTC13650	*Litoria verreauxii*	OR546031	y	Bungendore	NSW	-35.300	149.450		
ABTC13661	*Litoria verreauxii*	OR546017	y	Sassafras	NSW	-35.330	150.520		
ABTC13668	*Litoria verreauxii*	OR546032	y	Mulligans Flats	ACT	-35.150	149.150		
ABTC13674	*Litoria verreauxii*	OR546036	-	Numeralla Creek	NSW	-36.080	149.130		
ABTC17451	*Litoria verreauxii*	OR546037	y	Little Thedbo River, Thredbo Valley	NSW	-36.500	148.420	SAMA	R40219
ABTC17607	*Litoria verreauxii*	OR546038	y	7 km W Dalgety	NSW	-36.530	148.780	SAMA	R42617
ABTC24201	*Litoria verreauxii*	-	y	Watagan State Forest	NSW	-33.120	151.170	SAMA	R33929
ABTC24897	*Litoria verreauxii*	MT497827	-	Watagan State Forest	NSW	-33.120	151.170		
ABTC25248	*Litoria verreauxii*	OR546042	y	Barrington Tops	NSW	-32.040	151.500		
ABTC25255	*Litoria verreauxii*	OR546043	y	Mernot State Forest	NSW	-31.680	151.580		
ABTC25275	*Litoria verreauxii*	OR546044	y	Dingo Tops	NSW	-31.660	152.130		
ABTC86385	*Litoria verreauxii*	OR546039	-	Myrtleford	Vic	-36.597	146.767	ANWC	A02604
ABTC99134	*Litoria verreauxii*	-	y	Marthavale	Vic	-37.400	147.600		
AMSR186628	*Litoria verreauxii*	OR546033	-	Orroral River	ACT	-35.628	148.958	AMS	AMSR186628
AMSR188141	*Litoria verreauxii*	OR546045	-	Yetholme	NSW	-33.414	149.806	AMS	AMSR188141
AMSR188159	*Litoria verreauxii*	OR546034	-	Darkes Forest	NSW	-34.241	150.938	AMS	AMSR188159
AMSR188369	*Litoria verreauxii*	-	y	Tubbut	Vic	-37.040	148.558	AMS	AMSR188369
NMVZ13753	*Litoria verreauxii*	-	y	Dixons Creek	Vic	-37.608	145.376		
NMVZ57713	*Litoria verreauxii*	-	y	Dutson	Vic	-38.220	147.275	NMV	D 77286
NMVZ60371	*Litoria verreauxii*		y	Wombat Creek	Vic	-37.771	148.243	NMV	D 77302
NMVZ60407	*Litoria verreauxii*	-	y	Holey Plains State Park	Vic	-38.248	147.035	NMV	D 77813
ABTC139703	*Litoria watsoni*	MT497850	-	Mount Jersey Road, 14.k SSW Bonang	Vic	-37.292	148.643		
ABTC139706	*Litoria watsoni*	MT497849	-	Yalmy Road, 5.2k SSW Bonang	Vic	-37.226	148.704		
ABTC140298	*Litoria watsoni*	MT497846	-	Flat Rock Creek, Parma Creek Nature Reserve	NSW	-35.028	150.499	AMS	R177178
ABTC149194	*Litoria watsoni*	MT497845	-	Gerringong Falls	NSW	-34.661	150.654		
ABTC17597	*Litoria watsoni*	MT497848	-	5 km NE Tianjara Falls	NSW	-35.100	150.370	SAMA	R42607

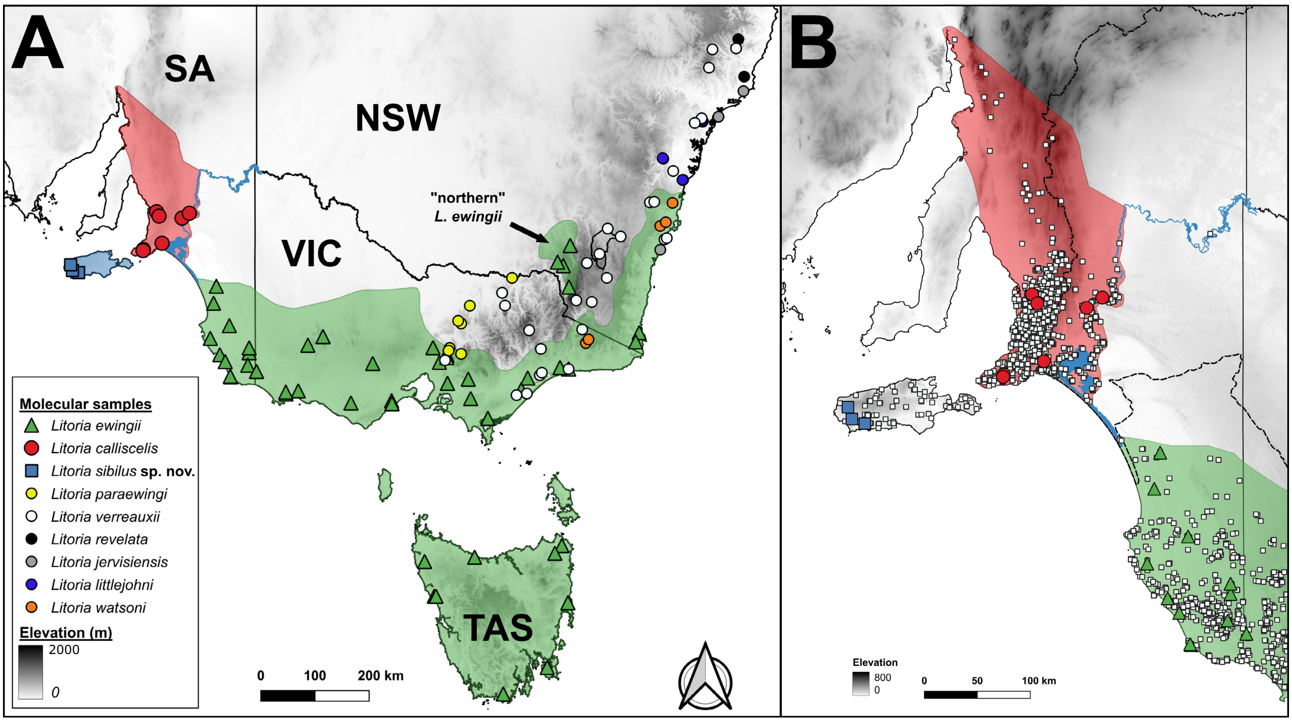

FIGURE 1. A) Map showing distribution of genotyped *Litoria ewingii* Group samples. South Australia inset (**B**) includes records from the Atlas of Living Australia and FrogID (small white squares). Murray River Basin indicated by dashed black line.

Net average sequence divergence between lineages (dA) was calculated in MEGA v7 (Kumar *et al*. 2016) as: dA = dXY–(dX + dY)/2, where, dXY is the average distance between groups X and Y, and dX and dY 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).

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). Reports for the Diversity Arrays orders were: DLit22-6849; DLit21-6068; DLit19-4642; DLit18-3455 and DLit17-2716.

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 (reproducibility) were chosen for subsequent analysis. Further filtering was undertaken on the basis of having a call rate <98% (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 that has been shown to perform well with codominant markers such as SNPs. We used the uncorrelated allele frequency, and the admixture ancestry models with prior locality information to assess values of K from 1 to 5. We performed 3 independent runs with 20,000 burnin and 50,000 MCMC iterations for each value of K. The preferred value of K was determined using the change in the second order of likelihood, ΔK (Evanno *et al.* 2005) in Structure Harvester webserver (Earl 2012). We then ran 10 independent runs with the preferred K for 20,000 burn-in 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 phylogenetic tree building methods suited to SNP data, SVDquartets and maximum likelihood. SVDquartets (Chifman & Kubatko 2014) accounts for differences in the genealogical histories of individual loci and for sequence variability due to both mutational and coalescent variance. In addition, the method is rapid and results are straightforward to interpret. A large number of quartets must be sampled to estimate phylogenetic relationships. Three independent runs of SVDquartets with sampling of all possible quartets were conducted in the program PAUP* version 4.0a build 165 (Swofford 2003) to assess topological convergence, each of which included 100 bootstrap replicates. We included representative samples of all eastern members of the *Litoria ewingii* Group in the phylogenetic analyses. To minimise distortion of phylogenetic estimates, individuals from known contact zones were excluded from the analysis.

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

Adult morphology. We examined preserved specimens held in the collections of the Australian Museum (AMS) and South Australian Museum (SAMA) and examined (either physically or via high-resolution images) all relevant name-bearing type specimens, including *Hyla ewingii* (Duméril & Bibron, 1841), *Hyla parvidens* (Peters, 1874), *Hyla calliscelis* (Peters, 1874), *Hyla inguinalis* (Ahl, 1935) and *Hyla ewingi iuxtaewingii* (Copland, 1957). We measured 24 morphometric characters (Table 2) for 65 specimens and documented variation in several external morphological traits considered to be potentially diagnostic (i.e., toe-webbing and aspects of colour/pattern). Sex and maturity of adults was determined by observing the presence of testes and darkened nuptial pads or vocal sacs in males, or the presence of oviducal follicles in females. Morphometric traits were measured with digital callipers to the nearest 0.1 mm from adult preserved specimens under microscope to ensure accurate placement of the callipers. Traits were adapted from the definitions of Watters *et al.* (2016).

To compare differences in geometric shape between taxa, we used a multivariate linear discriminant function analysis (DFA). Male and female samples were analyzed independently. Potentially confounding variation associated with differing body sizes and allometric growth was minimized by adjusting measurements to the values they would assume if they were of a mean body size for that sex using the allometric growth equation of Thorpe (1975): $Y_i^* = log_{10}Y_i – b(log_{10}SVL_i – log_{10}SVL_{mean})$, where Y_i^* is the adjusted value for character Y of the ith specimen; Y_i is the raw/unadjusted value for character Y; b is the mean of the regression coefficients for Y_i against SVL_i estimated independently for each taxon from logarithmically transformed values of Y_i and SVL_i; SVL_i is the measured snout-vent length (SVL) of the ith specimen; and SVL_{mean} is the pooled mean SVL.

DFA's were conducted after they had been adjusted for size/growth and log-transformed as described above using the 'lda' function from v7.3-40 of the R package MASS in RStudio v 4.2.1. We allocated specimens into three "taxa" for the DFA based on collection location. Prior group membership for specimens was established by choosing those that had either been genotyped or whose collection location fell within the range of each putative taxon. The raw mensural data and DFA results are presented in Supplementary Table S1, https://zenodo.org/record/8423599).

TABLE 2. Definition of morphometric traits measured for the *Litoria ewingii* Group. Asterisks indicate characters not defined by Watters *et al*. (2016).

Morphometric character	Definition
SVL	*Snout-vent length*. Direct line distance from tip of snout to posterior margin of vent.
HL	*Head length*. From the posterior of the jaws to the tip of snout.
HW	*Head width*. At the widest point; angle at the jaws.
HDD*	*Head depth*. from posterior edge of eye to directly under jaw.
IOD	*Interorbital distance*. The shortest distance between the anterior corners of the orbits.
DFE*	*Frontal eye distance*. Shortest distance between anterior edge of orbits, closest to snout.
IND	*Internarial distance*. Shortest distance between anterior edge of nostrils.
ED	*Eye diameter*. Horizontally from the anterior to the posterior corner of the eye.
SL	*Snout length*. Distance from the tip of the snout to the anterior corner of the eye.
EN	*Eye-nostril distance*. From anterior corner of the eye to the posterior margin of the nostril.
NS	*Snout-nostril length*. Distance from the centre of the external nares to the tip of the snout.
TL	*Tibia length*. Distance from the outer surface of the flexed knee to the heel/tibiotarsal inflection.
FOL*	*Foot-heel length.* Distance from tip of Toe 4 to heel/tibiotarsal inflection.
THL	*Thigh length*. Distance from the vent to the knee.
FL	*Foot length*. From base of the inner metatarsal tubercle to the tip of Toe 4.
IMT	*Inner metarsarsal tubercle length.* The greatest length of the inner metatarsal tubercle
HAL	*Hand length*. From the base of the outer palmer tubercle to the tip of Finger 3.
LAL	*Lower arm length.* Distance from elbow to the tip of Finger 3.
UAL	*Upper arm length.* From the body to the elbow.
AL*	*Arm length*. From the elbow to the tip of Finger 3.
Toe4W*	*Toe 4 width.* The greatest horizontal distance between edges of Toe 4, measured immediately behind the toe disk.
Toe4DW	*Toe 4 disk width*. The greatest horizontal distance between the edges of Toe 4 disk.
Fin3DW*	*Finger 3 disk width*. The greatest horizontal distance between edges of Finger 3 disk.
Fin3W*	*Finger 3 width.* The greatest horizontal distance between edges of Finger 3, measured immediately behind the finger disk.

Advertisement Calls. To examine variation in advertisement calls we obtained recordings from the FrogID citizen science database (Rowley *et al.* 2019, www.frogid.com.au). At the time of data export (August 2021), there were over 12,000 audio recordings of *Litoria ewingii* available. To obtain a subsample of quality recordings to analyse from across the range of each proposed taxon, we filtered the recordings in two phases. First, we removed duplicate recordings from single latitude and longitude locations, minimising the chances of resampling the same frog. In doing this, we preferentially selected recordings with a single species calling and those that had been marked as high quality in the database. We then visually inspected the locations of all recordings and filled any spatial gaps by selecting recordings with more than one species calling (i.e., species other than the target species). The second filtering phase involved removing recordings that were unsuitable for bioacoustics analysis. These calls typically did not contain a complete call, had too much background noise or had too many overlapping calls to accurately measure call parameters.

We converted FrogID recordings from MPEG AAC audio files into a WAV format (sampling rate of 48 kHz, 16 bits/sample) using the R package "av" prior to analysis in Raven Pro v1.6.3 (Center for Conservation Bioacoustics 2019). We measured call duration (s), note duration (s), dominant frequency (Hz), notes per call, pulses per notes, note rate (notes/s), pulse rate (pulses/s), note shape, frequency modulation, pulse pattern, pulse rate pattern, frequency pattern and the positions of the longest and shortest notes. Note shape refers to the shape of the note when viewed as a waveform (amplitude variation over time) and is divided into five categories in this study: wedge (amplitude increases with each pulse), tent (amplitude peaks near the middle of the note), wave (amplitude relatively consistent then increases towards end of the note), flat (note with relatively little variation in amplitude) or other (amplitude variations that did not fit the aforementioned criteria). We selected these call parameters based upon their utility in distinguishing the calls of frog species (Lötters *et al.* 2009, Forti *et al.* 2016, Köhler *et al.* 2017), including the *Litoria ewingii* Group (Littlejohn 1976, Littlejohn & Watson 1983, Smith *et al.* 2013, Sanders 2021). Based on the recommendations of Köhler *et al.* (2017), we calculated dominant frequency, pulse rate and pulses per note for each individual note within a call and then averaged these values across the call. In previous research (i.e., Watson *et al.* 1971, Watson 1972, Watson & Littlejohn 1978, Watson *et al.* 1985) values were calculated from a single note per call (i.e., 4th note of each call), however, given the calls of *Litoria ewingii* consist of between 8–30 notes, we felt this method would limit our ability to capture information from across the entire call. We used a fast-Fourier transformation of 512 points and 50% overlap.

To examine the variation between the taxa we grouped recordings based on their geographic proximity to genotyped samples and conducted one-way ANOVAs with Tukey's post hoc tests for honest significant differences. The raw acoustic data and locations of the recordings analysed are provided in Supplementary Table S2 (https://zenodo.org/record/8423599).

Conservation Assessments. To assess the conservation status of *Litoria calliscelis* and *L. sibilus*, we followed Criterion B of the IUCN Red List guidelines (IUCN 2022) and assessed the geographic range of each taxon through calculation of their area of occupancy (AOO) and extent of occurrence (EOO). *Litoria ewingii* was not assessed given its substantially widespread distribution (>200,000 km^2), relative abundance in FrogID submissions, and a lack of evidence for population decline or fragmentation. AOO and EOO calculations were made using the Atlas of Living Australia (ALA) spatial portal (www.ala.org.au, accessed online August 2022). For the assessments, we traced distribution polygons for each species in QGIS (v 3.10.9) using occurrence records obtained from ALA (accessed August 2022) and FrogID (up to August 2021) as a guide. These distribution polygons were then uploaded into the ALA spatial portal and used as the basis for assessment of AOO (2 x 2 km grid resolution) and EOO (minimum convex hull).

Under the IUCN Red List guidelines, to qualify as threatened under Criterion B, taxa must not only meet the minimum distribution threshold (AOO: <2,000 km^2, EOO: <20,000 km^2) but also at least two of three other conditions, specifically: (a) severely fragmented or number of locations ≤10; (b) continuing decline observed, estimated, inferred, or projected in any of: (i) EOO, (ii) AOO, (iii) area extent and/or quality of habitat, (iv) number of locations or subpopulations, (v) number of mature individuals; or (c) extreme fluctuations in any of: (i) EOO, (ii) AOO, (iii) number of locations or subpopulations, (iv) number of mature individuals.

Results

Molecular Genetic Analyses. The *ND4* sequences in the alignment varied in length from 641–821 bp. The phylogenetic analyses revealed strong support for three primary clades within *Litoria ewingii sensu lato*: (1) *L. sibilus*, (2) *L. calliscelis* and (3) widespread *L. ewingii* (Fig. 2). Net average sequence divergence between the taxa ranged from 6–9% (Table 3). A relatively low level of divergence (3%) was evident between mainland and Tasmanian samples of *Litoria ewingii.* In the *ND4* sequence alignment, *L. ewingii* is diagnosed by apomorphic nucleotide states at 8 sites, *L. calliscelis* by 10 and *L. sibilus* by 15 diagnostic sites (Table 4).

The total SNP dataset comprised 125,793 polymorphic SNP loci scored for 99 individuals. After filtering, 1,169 loci were retained with a total of 1.82% missing data. In the PCoA clustering analysis, the proportions of variation explained by the PC axes were: 1st axis—30.1%, 2nd axis—13.2%, 3rd axis—5.9% and 4th axis—5.2%. In the PCoA, samples of *Litoria ewingii* from Tasmania and the mainland clustered together, while *L. calliscelis* and *L. sibilus* formed discrete clusters, clearly separated from the *L. ewingii* cluster on the PC2 axis. Samples of a disjunct population of *Litoria ewingii* from northern Victoria and adjacent southern New South Wales, herein referred to as "northern *L. ewingii*", clustered between *L. ewingii*/*paraewingi* and *L. verreauxii* (Fig. 3A). The STRUCTURE analyses using a balanced sub-sample from the "in-group dataset" found K=3 as the optimal ancestry model. The three clusters corresponded to *Litoria calliscelis*, *L. sibilus* and *L. ewingii* (with mainland and Tasmanian samples combined) (Fig. 3B). The number of loci showing fixed allelic differences between clades used in the PCoA analyses ranged from 72–1146, with all values significant after simulation, except for the comparison between northern *Litoria ewingii* and *L. paraewingi* (Table 5).

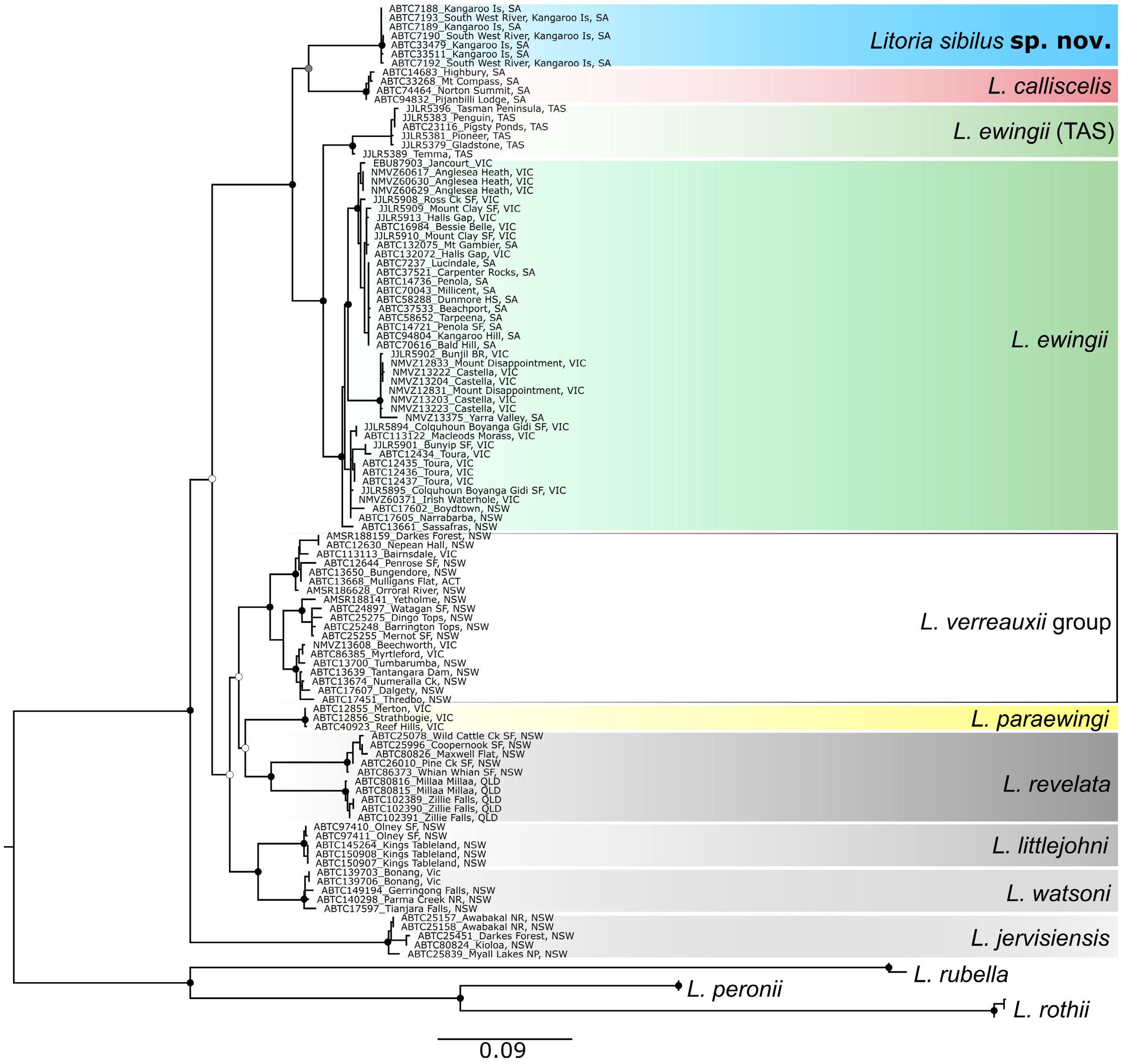

FIGURE 2. Maximum likelihood mtDNA (*ND4*) phylogeny of the *Litoria ewingii* Group inferred using IQ-TREE. Dots at nodes indicate bootstrap support values: >98% black dots; 70–97% grey dots; <70% white dots.

The phylogenetic analyses of the SNP dataset (Fig. 4) found well-supported splits between *Litoria sibilus*, *L. calliscelis* and *L. ewingii*, consistent with the mtDNA phylogeny.

Adult morphology. There was considerable overlap in raw morphological measurements between taxa for all characters measured so we relied on the Discriminant Function Analyses (DFA) for detecting variation in overall geometric shape between the taxa. The DFA's for males and females each returned two Linear Discriminant Functions (LD) (Fig. 5). For males (n = 39) and females (n = 26) the overall predictive accuracy was 100% and with jack-knifed validation, the classification success was 0.38 for males and 0.2 for females. For males, the traits with the highest coefficients for each of the two linear discriminants were, for LD1: Toe4W, HL, TL and Fin3DW and for LD2: Toe4DW, HL and EN. For females, the traits with the highest coefficients for each of the two linear discriminants were for LD1: HAL, TL and ED, and for LD2: FOL and Toe4DW.

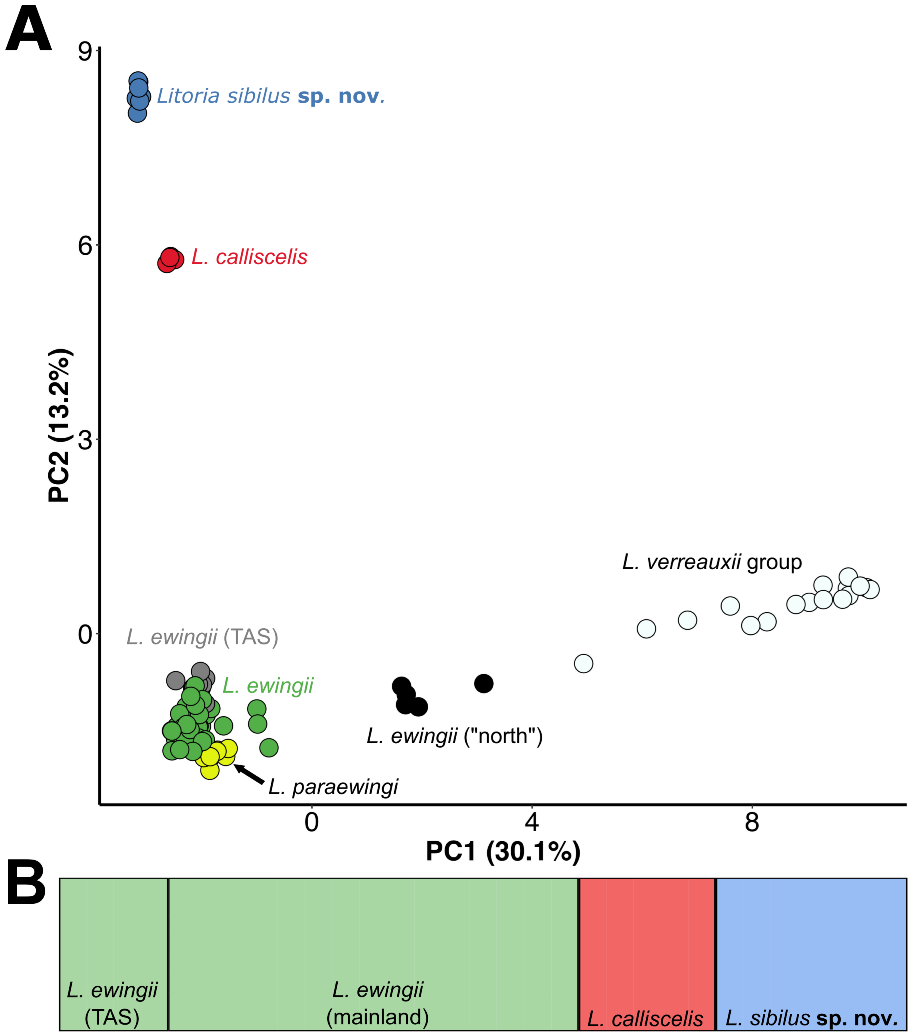

FIGURE 3. Clustering analyses of SNP data for the southern *Litoria ewingii* “in-group”: **A**) PCoA ordination plot, and **B**) STRUCTURE barplot for K=3 ancestry model.

TABLE 3. Net average sequence divergence between taxa (dA) of the *Litoria ewingii* Group. Values in bold are between sister lineages.

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *sibilus* **sp. nov.**	-													
2. *calliscelis*	**0.06**	-												
3. *ewingii* (Tasmania)	0.09	0.06	-											
4. *ewingii*	0.07	0.06	0.03	-										
5. *verreauxii*	0.09	0.08	0.07	0.07	-									
6. *paraewingi*	0.11	0.10	0.09	0.09	0.05	-								
7. *revelata* (north Qld)	0.11	0.10	0.10	0.10	0.07	0.08	-							
8. *revelata*	0.12	0.12	0.11	0.11	0.07	0.08	**0.08**	-						
9. *jervisiensis*	0.13	0.13	0.13	0.11	0.10	0.12	0.13	0.13	-					
10. *watsoni*	0.10	0.10	0.09	0.09	0.06	0.06	0.10	0.09	0.12	-				
11. *littlejohni*	0.12	0.11	0.11	0.11	0.06	0.08	0.10	0.09	0.13	**0.05**	-			
12. *peronii*	0.19	0.18	0.17	0.17	0.18	0.18	0.19	0.19	0.19	0.19	0.21	-		
13. *rothii*	0.20	0.20	0.19	0.20	0.20	0.22	0.21	0.21	0.22	0.21	0.22	0.18	-	
14. *rubella*	0.22	0.22	0.21	0.20	0.20	0.21	0.21	0.21	0.21	0.21	0.22	0.22	0.23	-

TABLE 4. Apomorphic diagnostic nucleotide states at 32 sites (in bold) for *Litoria ewingii*, *L. sibilus* **sp. nov.** and *L. calliscelis*. Sites numbers reflect position of the state within the *ND4* sequence alignment analysed.

	1	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3	3	3	3	4	5	5	6	6	6	6	6	6	6	6	6	6
	0	1	4	4	5	5	2	5	6	7	8	8	2	3	4	6	8	8	8	1	8	9	0	1	3	5	6	6	7	8	8	9
Taxon	7	0	0	9	5	8	5	7	0	8	1	7	3	2	7	8	1	3	9	0	7	3	2	4	3	7	2	3	5	3	7	5
L. sibilus **sp. nov.**	**C**	C	**C**	T	T	**C**	G	T	**G**	**T**	**C**	**C**	T	**C**	**C**	C	**G**	**C**	**C**	T	T	A	**G**	A	A	A	A	C	A	**G**	**G**	A
L. calliscelis	T	**T**	T	**C**	**A**	G	**A**	T	A	C	T	T	T	T	T	C	A	T	T	**C**	**C**	A	A	**G**	A	A	**G**	**T**	A	A	A	**G**
L. ewingii (mainland)	T	C	T	T	C/ T	G/ A	G	**C**	A/G	C	T	T	**C**	T	T	**T**	A	T	T	T	T	**G**	**G**	A	**G**	**G**	A	C	**G**	A	A	A
L. paraewingi	T	C	T	T	T	A	G	T	A	C	T	T	T	T	T	C	A	T	T	T	T	A	A	A	A	A	A	C	A	C	A	G
L. verreauxii	T	C	T	T/ C	C/ T	A	G	T	A	C	T	T	T	T	T	C	A	T	T	T	C/ T	A	A	A	A	A	T	C	A	T	A	A
L. watsoni	T	C	C	T	T	G	G	T	A	T	T	T	T	T	T	C	A	T	T	T	T	A	A	T	A	A	A	C	A	C	A	A
L. littlejohni	T	C	C	T	T	A	G	T	A	C	T	T	T	T	T	C	A	C	T	T	T	A	A	T	A	A	A	C	A	C	A	A
L. revelata	C	C	T	C	T	A	G	T	G	C	C	T	C	T	C	C	A	T	T	T	T	A	A	A	A	A	A	C	A	C	A	T
L. jervisiensis	T	T	T	T	T	A	G	T	A	C	C	T	T	T	C/ T	T	A	C	C	T	C	A/ G	G	A/ G	A	A	A	C/ T	A	T	A	T

TABLE 5. Numbers of loci showing fixed differences between taxa of the *Litoria ewingii* Group (upper right matrix). Values in parentheses are sample sizes. Lower left matrix is the expected numbers of loci showing fixed differences between taxa from simulations. Values in bold were significant after simulation.

Taxon	1	2	3	4	5	6
1. *ewingii* (47)	-	**613**	**482**	**196**	**72**	**66**
2. *sibilus* **sp. nov.** (7)	44	-	**724**	**1146**	**961**	**1012**
3. *calliscelis* (5)	39	63	-	**988**	**840**	**876**
4. *ewingii* ("northern") (5)	72	209	208	-	**539**	153
5. *ewingii* (Tasmania) (9)	25	124	116	225	-	**356**
6. *paraewingi* (8)	18	156	148	133	158	-

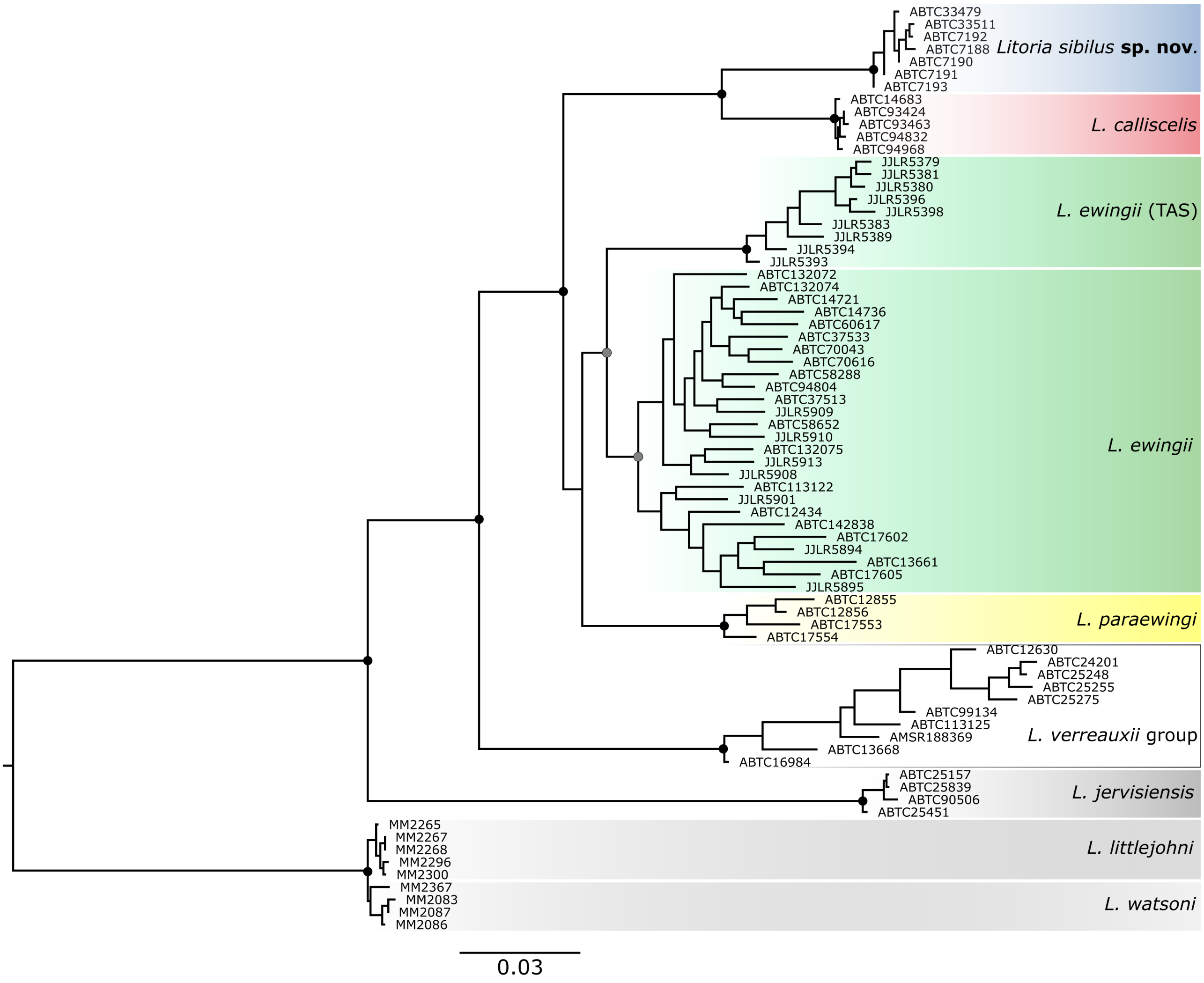

FIGURE 4. Maximum Likelihood phylogeny based on the concatenated SNP dataset for the eastern *Litoria ewingii* Group, with bootstrap support values indicated by dots at nodes (black = >98% support; grey = 70–98% support).

Advertisement Calls. The first phase of filtering resulted in 740 recordings retained, with the second phase retaining advertisement calls for 285 individual frogs suitable for acoustic analysis, including 219 *Litoria ewingii* from 165 localities, 59 *L. calliscelis* from 49 localities and 6 *L. sibilus* from four localities. The geographic coverage of the calls analysed encompassed the range of each taxon (see Supplementary Table S2, https://zenodo.org/record/8423599, for details of collection localities examined). Overall, there was extensive variation found both within and between the species (Fig. 6, Table 7). However, while there were varying degrees of overlap in call parameters, significant differences were found between *L. ewingii* and *L. calliscelis* in call duration, note duration, notes per call, pulses per note, note rate, pulse rate, and frequency modulation (all p values < 0.001). Significant differences were observed between *L. ewingii* and *L. sibilus* in note rate ($p < 0.001$) and pulse rate ($p = 0.014$), and between *L. sibilus* and *L. calliscelis* in call duration and notes per call (both p values < 0.001). Further differences were observed between taxa in note shape (*Litoria ewingii*—wedge shape 51.6% of calls, *L. sibilus*—tent shape 50%, *L. calliscelis*—other shape 27%), positions of the longest notes (*L. ewingii*—beginning 85%, *L. sibilus*—end 50%, *L. calliscelis*—end 71%) and shortest notes (*L. ewingii*—other 44%, *L. sibilus*—beginning 83%, *L. calliscelis*—beginning 80%), the average number of notes per call (*L. ewingii*—9, *L. sibilus*—9, *L. calliscelis*—16) and average call duration (*L. ewingii*—2.4s, *L. sibilus*—1.8s, *L. calliscelis*—3.3s). Secondary differences (i.e., those which were less consistent in differentiating the species) included average note duration (*L. ewingii*—223ms, *L. sibilus*—166ms, *L. calliscelis*—160ms), average note rate (*L. ewingii*—3.3 notes/s *L. sibilus*—4.8 notes/s, *L. calliscelis*—4.5 notes/s) and average pulse rate (*L. ewingii*—106.4 pulses/s, *L. sibilus*—112.2 pulses/s, *L. calliscelis*—112.2 pulses/s). Dominant frequency did not differ substantially between the taxa.

Systematic Implications. We conclude *Litoria ewingii sensu lato* comprises three distinct lineages which constitute separate species under the evolutionary species concept (*sensu* de Quieroz 1998). Evidence for lineage separation between these taxa is supported by concordance between our mtDNA and SNP datasets, specifically:

1. The presence of three well-supported, reciprocally monophyletic mtDNA and SNP clades in the phylogenetic analyses.
2. A level of mtDNA sequence divergence between the taxa ranging from 6–9%, which is equal or greater than that of other well-accepted species pairs within the *Litoria ewingii* Group (Table 3)
3. The accumulation of a significant number of SNP loci showing fixed allelic differences between the lineages (Table 5), which is a robust indication of long-term reproductive isolation between the taxa (Georges *et al.* 2018).
4. Bayesian and PCoA clustering analyses of the SNP data consistently identify three distinct genetic clusters, with no admixture between them.

Further, despite overlap in advertisement call structure between the taxa, we found evidence for divergence in numerous call parameters, including call duration, note duration, notes per call, pulses per note, note rate, pulse rate, frequency modulation, note shape and positions of the longest and shortest notes. Although no single consistent morphological character was found to diagnose these taxa from each other, they may be identified using a combination of characters. In addition, our DFA for detecting variation in geometric shape demonstrated a high degree of predictive accuracy (100%), indicating the taxa are morphologically divergent, albeit subtly.

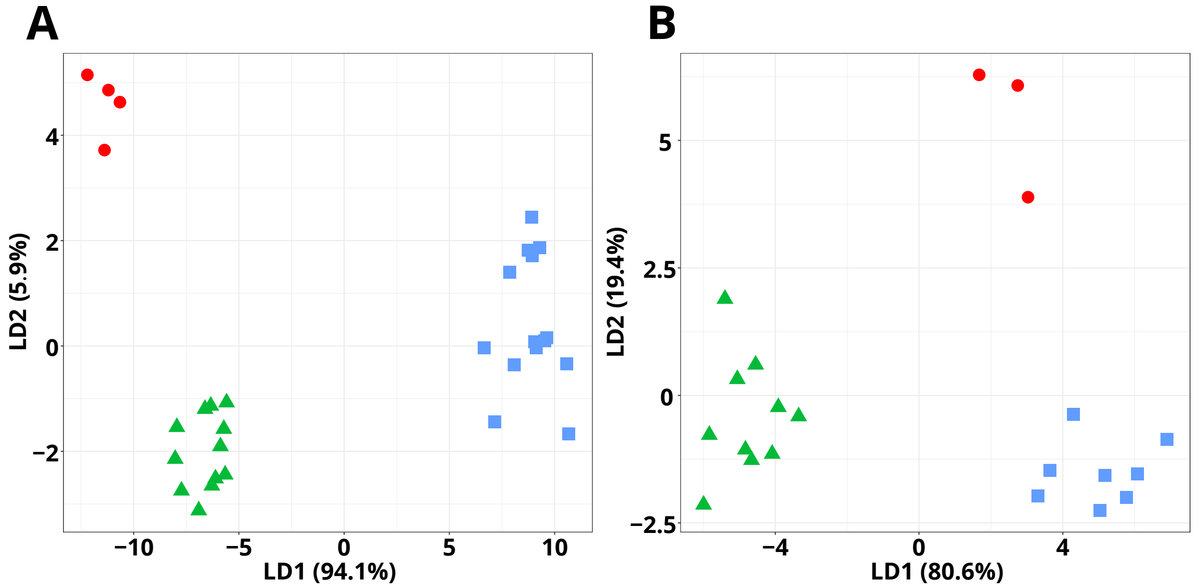

FIGURE 5. Results of linear discriminant function analysis of adult morphometric characters in *Litoria ewingii* Group, **A**) males, and **B**) females. Green triangles = *Litoria ewingii*, red circles = *Litoria calliscelis*, blue squares = *Litoria sibilus* **sp. nov.**

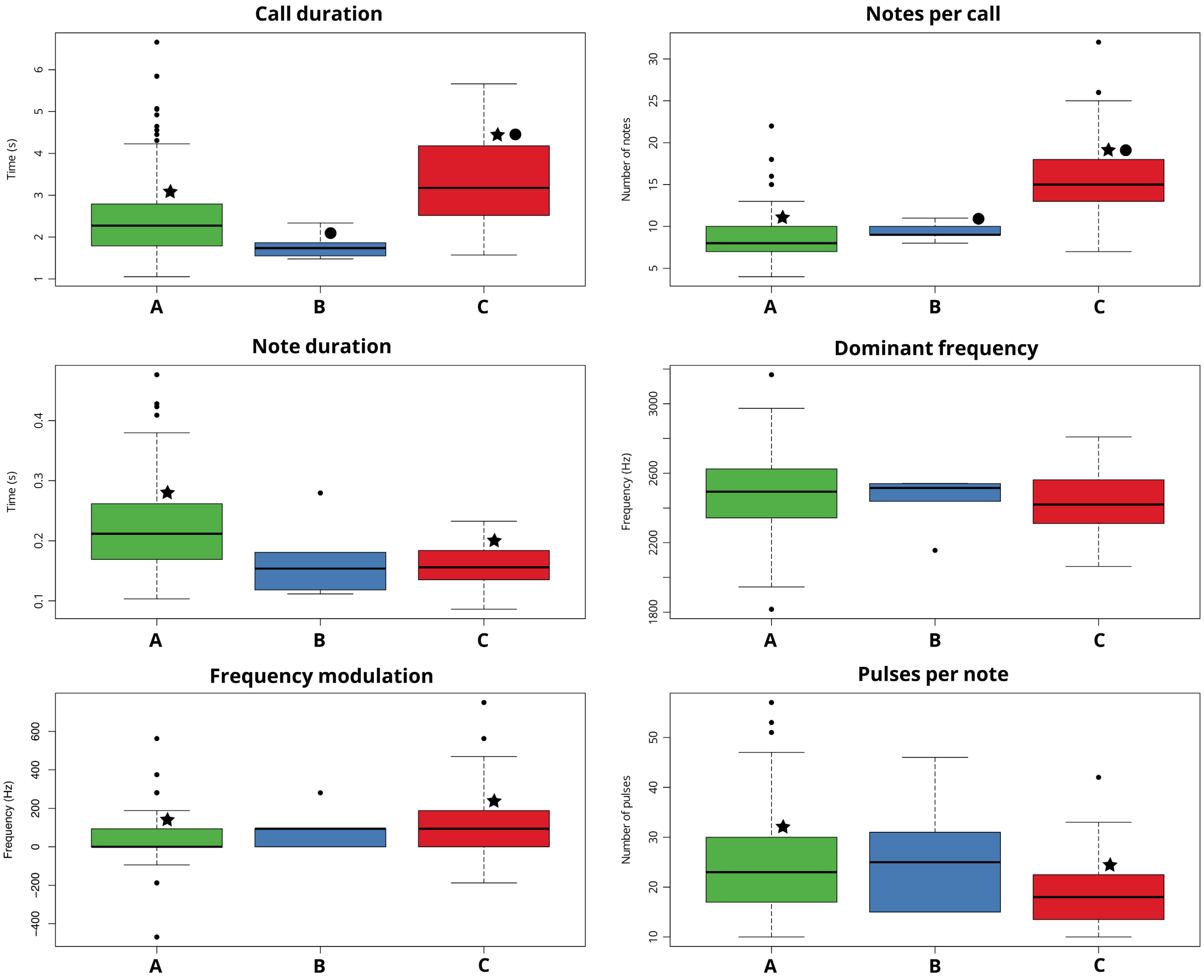

FIGURE 6. Comparison of advertisement call parameters between species of the *Litoria ewingii* Group. **A**) *Litoria ewingii* (n=219 individual frogs from 165 localities), **B**) *Litoria sibilus* **sp. nov.** (n=6 from four localities), and **C**) *Litoria calliscelis* (n=59 from 49 localities). Symbols beside boxplots denote parameters that were significantly different between pairs of taxa.

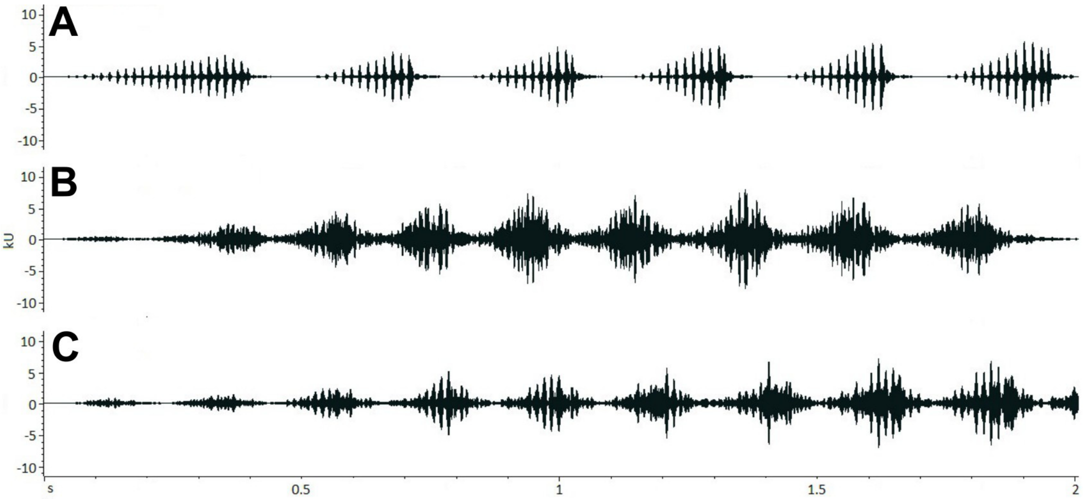

FIGURE 7. Comparison of audio spectrograms of the advertisement calls of **A**) *Litoria ewingii*, Lake Burbary, Queenstown, Tasmania (FrogID: 166278) **B**) *Litoria sibilus* **sp. nov.**, Pelican Lagoon, Kangaroo Island, South Australia (FrogID: 23471) and **C**) *Litoria calliscelis*, Victor Harbour, Fleurieu Peninsula, South Australia (FrogID: 242125).

The identity of specimens of the apparently disjunct northern *Litoria ewingii* population from north-eastern Victoria and the adjacent western slopes of New South Wales remains unclear. Previous assessments of the interactions between northern *Litoria ewingii* and adjacently distributed *L. paraewingi* and *L. verreauxii* indicate hybrid zones are present where the taxon ranges abut (Watson et al 1971, Watson & Littlejohn 1978). Our genetic analyses appear to concur that specimens from this region are intermediate between *Litoria ewingi*/*L. paraewingi* and *L. verreauxii*. Our analyses of fixed allelic differences show the comparison between northern *Litoria ewingii* and *L. paraewingi* was not significant after simulation, highlighting a close genetic affinity between these taxa. Further investigation is required to evaluate the dynamics of interaction between northern *Litoria ewingii* and adjacent taxa to confirm its taxonomic status however, for now, we conservatively retain this population as conspecific with *L. ewingii*.

Taxonomy

Nomenclature. To evaluate the nomenclatural implications of our study, we examined high-resolution images of the *Hyla ewingii* Duméril & Bibron, 1841 lectotype (designated implicitly by Guibé, 1950) and all additional name-bearing type specimens considered to be junior synonyms of *Litoria ewingii sensu lato*.

The *Hyla ewingii* lectotype (MNHN 4851, type locality: Tasmania, Fig. 9) represents the earliest available name for the widespread taxon here referred to as *Litoria ewingii*. *Hyla parvidens* Peters, 1874 (Holotype: ZMB 8252, type locality: Port Phillip, Victoria) is generally assumed to be a junior synonym of *Litoria ewingii,* although Fletcher (1898) only alluded to synonymy without formally doing so. Our examination of images of the *Hyla parvidens* holotype confirm it corresponds in morphology with topotypic *Litoria ewingii* (toe pads wider than digits, extensive webbing [see Fig 8/Type B], absence of inguinal patterning) rather than the sympatrically occurring *L. verreauxii*, and so we formally place it in the synonymy of *L. ewingii*. Furthermore, we physically examined the type of another junior synonym, *Hyla ewingii iuxtaewingii* Copland, 1957 (Holotype: AMS R.85610, type locality: 18 miles W. of Bairnsdale, Victoria), previously synonymised with *Litoria ewingii* by Moore (1961). Around the vicinity of the *Hyla iuxtaewingii* type locality in western Victoria, *Litoria ewingii* and *L. verreauxii* occur in sympatry. Based on the external morphological traits of toe pad size, extent of webbing and inguinal patterning, we consider the *Hyla ewingii iuxtaewingii* holotype to be more closely aligned to *Litoria verreauxii*. The toe pads of the *Hyla ewingii iuxtaewingii* holotype are equal in size to the digits (Toe4W/Toe4DW = 1.0) similar to *Litoria verreauxii*, versus wider than the digits for *L. ewingii* (mean Toe4W/Toe4DW = 0.7), the toes are relatively basally webbed (extending to the 2nd subarticular tubercle on the 4th toe) more like *L. verreauxii* versus moderately extensive webbing in *L. ewingii* (usually extending from 1st—halfway between 1st and 2nd subarticular tubercle rarely extending to the 2nd [see Fig 8, Type B]), and there is distinct spotted pattern in the inguinal region of the specimen similar to *L. verreauxii*, which is typically absent in *L. ewingii* (n=29/33). Based on these morphological characters, we place *Hyla ewingii iuxtaewingii* in the synonymy of *Litoria verreauxii*.

The earliest available name for the taxon occurring in Adelaide, Fleurieu Peninsula and Mount Lofty Ranges, South Australia, is *Hyla calliscelis* Peters, 1874, represented by two syntypes (ZMB 8251; 92805) collected in Adelaide. Images of both types confirm the specimens correspond in morphology with topotypic individuals from the region and so we resurrect the name *Litoria calliscelis* for this taxon. In addition, we designate ZMB 92805 as the lectotype for *Litoria calliscelis* and provide a revised description of the specimen below. Finally, we consider the later name of *Hyla inguinalis* Ahl, 1935 to be a junior synonym of *Litoria calliscelis*. Based on type locality (Adelaide) and similarities in aspects of inguinal and thigh patterning present in the *Hyla inguinalis* holotype (ZMB 14080) and paratypes (ZMB 35201–35205), we formally place it in the synonymy of *Litoria calliscelis*.

There are no existing names in the taxonomic literature available for the taxon restricted to Kangaroo Island, so we herein describe it as a new species, *Litoria sibilus* **sp. nov.**

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

Group membership was also confirmed by the mtDNA phylogeny presented here and in Mahony *et al*. (2020).

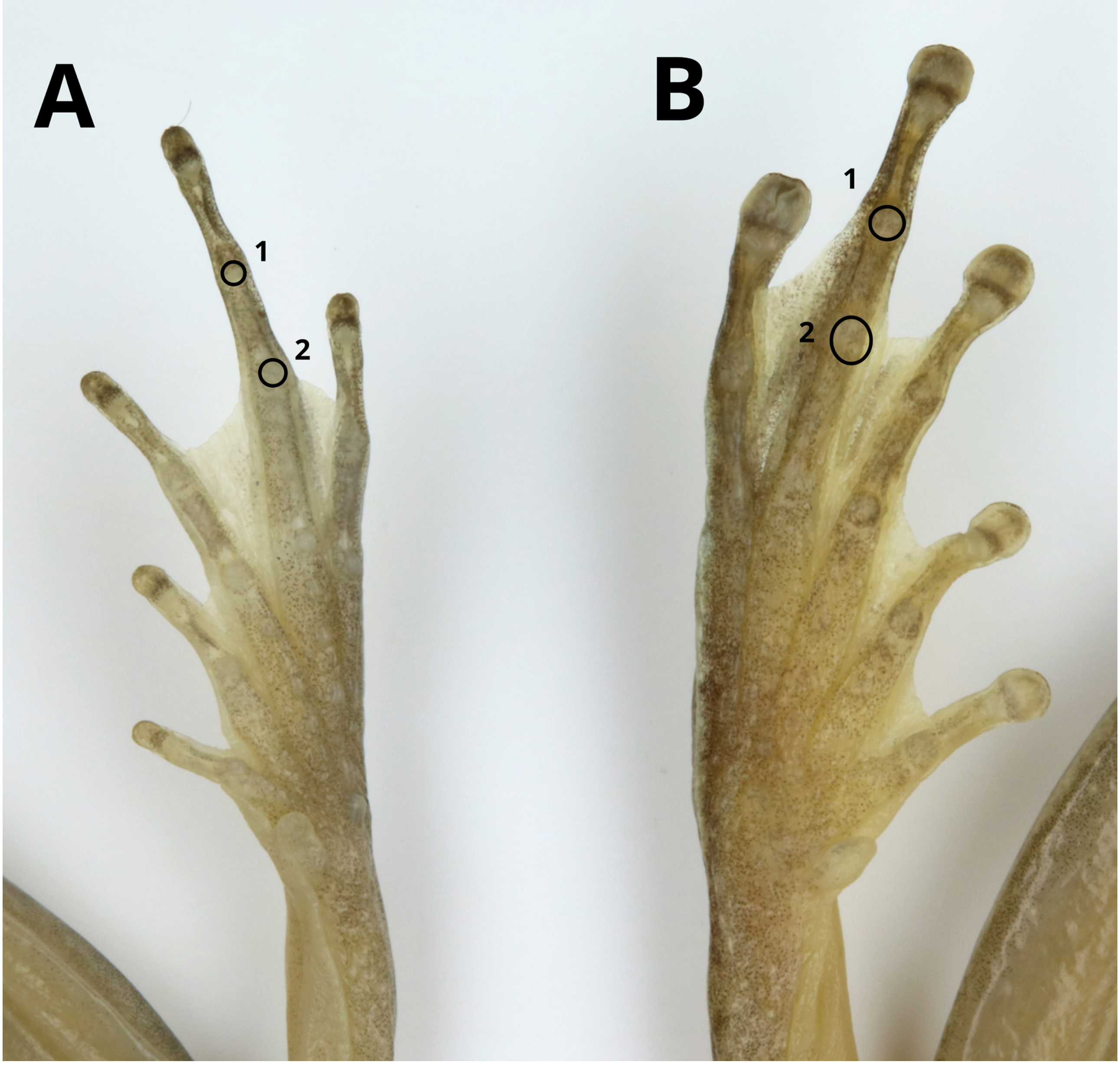

FIGURE 8. Comparison of the hind feet of *Litoria verreauxii* and *L. ewingii* showing differences in toe pad size and webbing extent: Type **A**) *Litoria verreauxii* and Type **B**) represents *Litoria ewingii, L. calliscelis* and *L. sibilus* **sp. nov.** Subarticular tubercles 1 and 2 are indicated by circles.

Litoria ewingii (Duméril & Bibron, 1841)

Suggested common name: Brown Tree Frog
Figs 9,10

Hyla ewingii. Duméril, A.M.C. & Bibron, G. (1841) Erpétologie Genérale ou Histoire Naturelle Complète des Reptiles. Volume 8. Paris: Librarie Enclyclopedique de Roret

Lectotype. MNHN 4851 (designated by Guibe, 1950). Type locality: "*la terre de Van Diémen*" = Tasmania, Australia.

Synonyms.

Hyla parvidens. Holotype: ZMB 8252. Type locality: "Australien (Port Phillip)", Victoria, Australia. Peters, W.C.H. (1874) Über neue Amphibien (*Gymnopis, Siphonops, Polypedates, Rhacophorus, Hyla, Clyclodus, Euprepes, Clemmys*). *Monatsberichte der Königlichen Preussische Akademie des Wissenschaften zu Berlin,* 1874, 616–624.

Material examined. *Hyla ewingii* and *Hyla parvidens* types viewed from high-resolution images. See Supplementary Table S1 (https://zenodo.org/record/8423599) for full list of specimens used in morphometric analyses.

Revised diagnosis. *Litoria ewingii* is diagnosable from all other members of the *ewingii* group based on a combination of (1) adult body size 28–35 mm for males, 28–45 mm for females, (2) moderately robust build, (3) pads wider than fingers (mean Fin3W/Fin3DW = 0.6) and toes (mean Toe4W/Toe4DW = 0.7), (4) webbing vestigial on the hands but relatively well-developed on the feet (typically extending from 1st—halfway between 1st and 2nd subarticular tubercle, rarely extending to the 2nd [see Fig 8, Type B]), (5) posterior edge of thigh orange-yellow and usually plain, unpatterned (n=27/32), but sometimes with scattered dark spots, flecks or lines (n=5/32), (6) dark spots in the inguinal region usually absent (n=29/33), although occasionally present (n=4/33), (7) genetically by apomorphic nucleotide states at 8 sites in the *ND4* gene (Table 4). Diagnoses of *Litoria ewingii* and the other species described herein are presented in Table 8 for ease of comparison.

Redescription of Holotype. We redescribe the holotype based on images of the preserved specimen after more than 180 years in preservative (Fig. 9). Habitus moderately robust. Head slightly longer than wide, widest at commissure of the jaws. Tympanum smaller than eye, rounded and partially obscured by a tympanic fold. Snout rounded in dorsal and lateral profiles. Fingers unwebbed and toes with moderate webbing. Finger and toe pads wider than digits. Sub-articular tubercles visible, metacarpal tubercles prominent, inner-metatarsal tubercles somewhat prominent, oblong-shaped and approximately one-third the length of the fourth toe. Arms and legs moderately long and slender. Texture of dorsal surface finely granular, ventral surface coarsely granular, inguinal region, lower surface of tibia, upper surface of thighs and throat smooth.

Colour in preservative. Dorsum varies tonally between copper-brown—beige and cream with faded dark bifurcated markings running longitudinally from between eyes to vent. Ventral surface light coppery-yellow.

Variation. Summary of variation in morphometric characters for each sex is presented in Table 6.

Colour and pattern (in life). Variation in colour described from images taken in life (Fig 10). Dorsum base colour varies from cream, beige, grey to light copper brown, occasionally even lime green (i.e., Fig 10/D and F), often fading in intensity posteriorly. Darker grey to copper-brown longitudinally aligned and bifurcated (occasionally completely separated) bands usually extend from between eyes to vent, although may be faded or absent in some individuals. Upper surface of legs brown, cream to grey or green with posterior edge of thigh orange-yellow and typically unpatterned (n=27/32), but sometimes with scattered dark spots, flecks or lines (n=5/32). Light cream zone usually present on posterior edge of shoulder, with grey, pink or yellow in armpit. Brown, grey or silver stripe runs from rostrum through eye and fades into the lateral zone. Cream stripe typically runs from below eye to edge of mouth, often extending to the shoulder or elbow. Iris cream to coppery.

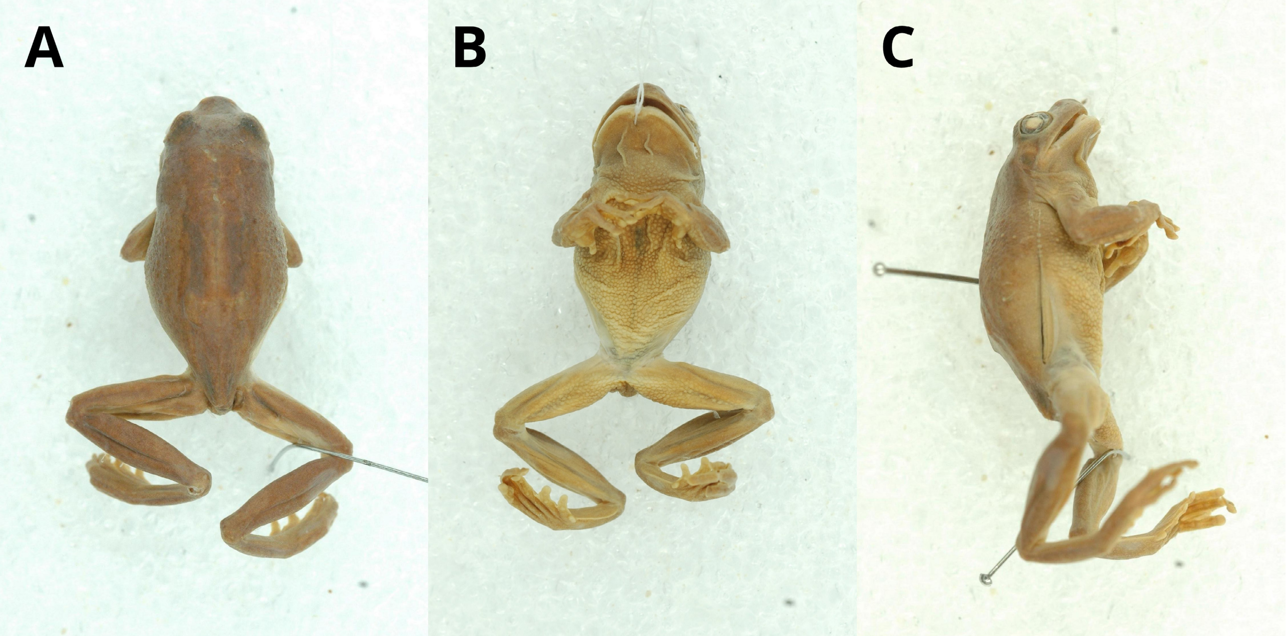

FIGURE 9. Images of the holotype of *Hyla ewingii*, MNHN 4851 in **A**) dorsal; **B**) ventral and **C**) lateral profile. Images obtained from the Muséum National d'Histoire Naturelle online portal (Frédéric Braux 2012).

TABLE 6. Summary of metric variation in morphological analyses for *Litoria ewingii*, *L. calliscelis* and *sibilus* **sp. nov.** Measurements are presented as the mean±SD and range.

Character	*Litoria ewingii*		*Litoria calliscelis*		*Litoria sibilis* **sp. nov.**	
	females	males	females	males	females	males
SVL	37.4±4.4	31.4±2.1	34.1±4.9	31.5±2.7	36.6±4.7	31.5±2.6
	28.1–44.8	28.5–35.5	27.8–40.3	27.4–33.7	27.9–42.7	25.1–34.5
Fin3DW	1.3±0.4	1.0±0.2	1.6±0.4	1.1±0.1	1.4±0.4	1.1±0.2
	0.9–2.2	0.7–1.2	1.2–2.2	1.0–1.3	1.0–2.0	0.7–1.4
Fin3W	0.8±0.2	0.6±0.1	0.8±0.1	0.7±0.1	1.0±0.2	0.7±0.2
	0.5–1.2	0.4–0.8	0.6–0.9	0.6–0.8	0.7–1.2	0.5–1.0
Toe4DW	1.2±0.3	0.9±0.2	1.4±0.4	1.0±0.2	1.4±0.4	1.0±0.2
	0.7–1.9	0.5–1.4	0.9–2.0	0.8–1.1	0.8–1.9	0.6–1.4
AL	17.8±2.4	14.9±1.2	16.7±2.9	15.1±1.3	18.3±2.8	14.7±1.5
	13.5–22.1	13.4–17.2	12.4–19.5	13.2–16.4	12.9–21.0	11.3–16.4
DFE	6.2±0.5	5.4±0.4	6.5±1.0	5.7±0.5	6.5±1.0	5.5±0.4
	5.0–7.1	4.9–6.3	5.7–8.0	5.0–6.1	4.6–7.4	4.4–5.9
EN	2.7±0.4	2.4±0.2	2.8±0.4	2.3±0.3	2.9±0.4	2.5±0.2
	2.1–3.4	2.1–2.8	2.2–3.2	1.9–2.6	2.1–3.5	2.3–3.1
ED	3.4±0.3	3.0±0.4	3.8±0.6	3.2±0.3	3.8±0.4	3.4±0.3
	2.6–3.7	2.3–3.7	3.0–4.7	2.8–3.5	3.1–4.5	3.0–4.1
THL	18.1±2.8	15.5±1.3	16.1±2.8	15.5±1.0	17.8±2.5	14.8±1.2
	13.3–22.8	13.5–17.6	12.1–19.7	14.4–16.9	12.9–21.1	12.4–16.9
FOL	26.6±3.7	22.7±1.9	25.1±4.3	22.6±2.0	27.1±4.4	22.2±2.3
	20.2–33.1	19.6–25.9	19.0–30.2	19.5–24.4	19.0–31.7	16.9–24.5
HDD	4.7±0.7	3.8±0.5	4.7±0.7	4.0±0.4	4.9±0.7	4.0±0.4
	3.3–6.1	2.8–4.5	3.7–5.4	3.4–4.4	3.8–6.2	3.5–4.9
HL	12.6±1.6	11.1±2.0	13.9±2.3	12.6±0.9	15.2±1.9	12.8±0.9
	10.3–15.8	9.0–14.8	10.7–16.2	11.1–13.4	11.8–17.5	10.9–13.9
HW	11.0±1.1	9.6±0.6	11.3±2.4	9.9±0.6	11.6±1.5	9.8±0.7
	9.1–12.3	8.6–10.7	8.3–14.6	9.2–10.9	8.5–13.1	7.9–10.6
IMT	1.3±0.2	1.1±0.2	1.3±0.4	1.5±0.2	1.2±0.3	1.0±0.2
	1.0–1.9	0.7–1.4	0.8–1.8	0.9–1.4	0.9–1.6	0.7–1.3
IND	2.5±0.3	2.4±0.2	2.6±0.3	2.3±0.1	2.6±0.3	2.3±0.1
	2.3–3.4	2.1–2.9	2.3–2.8	2.2–2.5	2.2–3.2	2.0–2.6
IOD	3.6±0.3	3.1±0.4	3.6±0.3	3.5±0.2	3.7±0.4	3.3±0.3
	2.9–4.0	2.4–3.6	3.2–4.1	3.2–3.6	3.1–4.3	2.8–3.7
LAL	7.9±1.1	6.8±0.5	7.5±0.7	6.9±0.5	8.3±1.5	6.9±0.6
	5.9–10.0	5.6–7.7	6.3–8.2	6.3–7.4	5.4–9.9	5.4–7.6
HAL	10.5±1.5	8.9±0.7	10.3±1.9	8.9±0.8	11.0±1.8	8.8±1.0
	7.4–13.0	7.9–10.2	7.9–12.5	7.9–9.7	7.7–12.7	6.6–10.0
NS	2.3±0.3	1.9±0.2	2.4±0.5	2.2±0.1	2.3±0.4	2.2±0.2
	1.6–2.7	1.5–2.2	1.6–3.0	2.0–2.4	2.0–2.9	1.8–2.6
FL	16.3±2.4	13.8±1.2	15.3±2.7	13.8±1.5	16.2±2.6	12.9±1.2
	12.2–20.4	11.9–16.3	11.8–18.2	11.6–15.3	11.2–19.2	9.9–14.1
SL	4.9±0.5	4.3±0.2	5.0±0.4	4.6±0.2	5.2±0.7	4.5±0.3
	4.1–5.7	3.8–4.7	4.3–5.5	4.5–4.8	3.7–5.9	4.0–5.0
TL	18.5±2.6	15.6±1.2	17.5±3.0	15.9±1.1	19.3±2.7	15.8±1.1
	13.9–23.1	14.0–18.3	13.3–20.5	14.4–17.2	14.4–22.9	13.4–17.4
UAL	6.5±0.6	5.4±0.9	5.9±1.0	5.8±0.6	6.7±1.4	5.6±0.7
	5.8–7.7	4.5–7.4	5.2–7.3	5.0–6.5	3.8–8.3	4.4–6.9

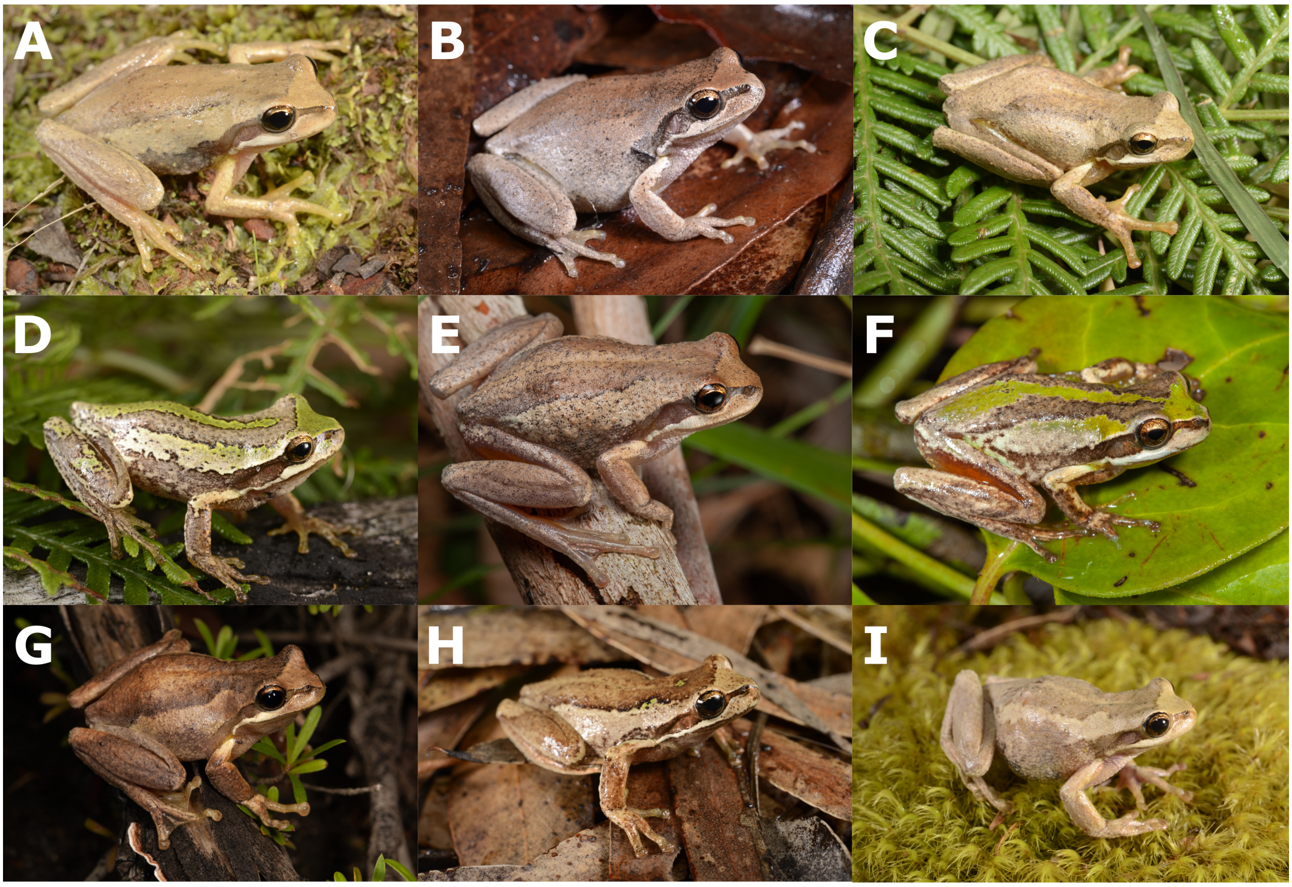

FIGURE 10. Images in life of *Litoria ewingii*. **A**) NMV D 76462 (JJLR5913), adult female, Halls Gap, Grampians, Victoria; **B**) NMV D 76454 (JJLR6056), Lyonville, Victoria. **C**) NMV D 76455 (JJLR5894), adult male, Colquhoun/Boyanga Gidi SF, south-eastern Victoria; **D)** NMV D 76461 (JJLR5910), adult male, Mount Clay State Forest, south-western Victoria; **E–G** unvouchered individuals photographed by Stephen Mahony; **E**) Naringal, Victoria; **F**) Mornington Peninsula, Victoria. **G**) Toongabbie, Victoria; **H**) TMAG C1531 (JJLR5389), adult male, Temma, Tasmania; **I**) TMAG C1533 (JJLR5394), adult female, Zeehan, Tasmania.

Advertisement Call. Call description is based on the calls of 219 individuals. The advertisement call of *Litoria ewingii* has a duration of 1.05–6.66 s (mean 2.42 s) and comprises 4–22 distinctly pulsed notes (mean 9) with most calls (85.4%) beginning with a long note followed by a series of shorter notes. Note duration is between 0.10–0.48 s (mean 0.22 s). In the majority of calls (51.6%), notes rise in amplitude, appearing as a wedge shape in waveform view with a short interval between notes (Fig. 7). Dominant frequency has a range of 1817–3167 Hz (mean 2485 Hz) and remains fairly stable throughout the call.

Comparisons with similar species. *Litoria ewingii* may occur in sympatry with *L. verreauxii* and *L. paraewingi* where identification can be challenging, particularly for hybrid individuals. It shares a broad zone of overlap with *Litoria verreauxii* in eastern Victoria and southern NSW where occasional hybridisation occurs due to mis-mating (Smith *et al.* 2012). In addition, *Litoria ewingii* shares a narrow hybrid "tension" zone with *L. paraewingi* in central Victoria (Watson *et al.* 1971, Smith *et al.* 2013). Non-hybrid *Litoria ewingii* can be readily distinguished from *L. verreauxii* using the following characters: finger and toe pads distinctly wider than digits (*L. ewingii* mean Fin3W/Fin3DW 0.6; Toe4W/Toe4DW 0.7 versus *L. verreauxii* mean Fin3W/Fin3DW 0.9; Toe4W/Toe4DW 1.0, n = 47), extensive webbing on the toes (*L. ewingii* webbing usually extends to the 1st or halfway between the 1st and 2nd subarticular tubercle on the 4th toe versus extending to the 2nd subarticular tubercle on the 4th toe for *L. verreauxii* [see Fig 8 for comparison]), and by an absence of patterning in the inguinal region (versus present in *L. verreauxii*). Distinguishing *Litoria ewingii* from *L. paraewingi* may be especially problematic where their distributions overlap, and mating-calls are unavailable. Watson *et al.* (1971) note in the original description of *Litoria paraewingi* that the species is morphologically indistinguishable from northern *L. ewingii*, however, *L. paraewingi* can be distinguished from southern populations of *L. ewingii* by smaller adult body size, relatively longer head and a straight versus rounded canthus rostralis.

Litoria ewingii is allopatric with *L. sibilus* and *L. calliscelis*, separated from the latter by a gap of approximately 80 km across the Coorong region and adjacent northern Naracoorte Coastal Plain. Dispersal across this landscape is likely hindered due to a lack of suitable breeding habitat—the Coorong is characterised by an expanse of saline coastal flats and the predominantly dry, mallee-dominated northern Naracoorte Coastal Plain is lacking in sufficient surface waters for breeding. Despite significant overlap in morphological characters between *Litoria ewingii*, *L. calliscelis* and *L. sibilus*, some aspects of colour/pattern may be useful for identification. *Litoria ewingii* usually have a plain, unpatterned posterior edge of the thigh (n=27/32), compared to thigh patterning comprising dark spots and blotches for *L. calliscelis* (n=10/11) and *L. sibilus* (n=26/27). *L. ewingii* can be further distinguished from *L. calliscelis* by a typical absence of inguinal patterning (n=29/33) versus inguinal pattern usually comprising a single dark spot at the junction of the thigh and abdomen (n=7/11).

Distribution and habitat. Widespread throughout south-eastern Australia including Tasmania and the south-eastern mainland coast, ranging from southern New South Wales, across Victoria, as far west as Deepwater in South Australia. An introduced population exists in New Zealand, believed to be introduced from Tasmania in 1875 (Bazin *et al*. 2007, Rexer-Huber *et al.* 2015). Occurs from near sea level to an elevation of at least 1200 m (Watson *et al.* 1985). Possesses a remarkable tolerance to sub-zero temperatures (Bazin *et al.* 2007, Rexer-Huber *et al.* 2015).

Considered a habitat generalist, *Litoria ewingii* occurs in a variety of habitats including wet and dry sclerophyll forests, heathland and highly disturbed agricultural and urban areas.

TABLE 7. Summary of acoustic variation in advertisement call parameters for *Litoria ewingii*, *L. calliscelis* and *L. sibilus* **sp. nov.** Measurements are presented as the range and the mean.

Call parameter	*L. ewingii*	*L. calliscelis*	*L. sibilus* **sp. nov.**
Number of calls (n)	219	59	6
Number of locations	165	49	4
Call duration (s)	1.053–6.657	1.571–5.663	1.479–2.336
	2.421	3.347	1.783
Note duration (s)	0.103–0.476	0.086–0.233	0.112–0.279
	0.223	0.16	0.166
Dominant frequency (Hz)	1817–3167	2063–2809	2156–2541
	2485	2444	2451
Notes / call	4–22	7–32	8–11
	9	16	9
Pulses / note	10–57	10–42	15–46
	24	19	26
Note rate (notes/s)	1.437–6.347	2.719–7.617	2.997–6.45
	3.271	4.549	4.823
Pulse rate (Pulses/s)	33.59–203.75	43.02–192.68	98.91–175.17
	106.35	112.18	147.04
Frequency pattern	Stable	Stable	Up and down
Note shape	Wedge	Other	Tent
Longest note	Beginning	End	End
Shortest note	Other	Beginning	Beginning

Ecology. The species is recorded commonly via FrogID (>18,000 records from 10 November 2017–30 June 2022), and is relatively commonly heard calling in disturbed areas, with 24% of FrogID records from urban habitats and 47% of records from rural areas. *Litoria ewingii* have been recorded calling year-round in association with rainfall, with a distinct peak July–November based on FrogID submissions. Breeding occurs in static ephemeral or permanent waterbodies, including dams, ponds, swamps, inundated ditches and streamside ponds and pools (Anstis

2017, Parkin *pers. obs*). For a detailed description of tadpole development and morphology, see Anstis (2017). Tadpoles are highly sensitive to increases in salinity (Chinanthamby *et al.* 2006).

Conservation status. Based on its widespread distribution (>200,000 km^2), relative abundance in FrogID submissions, and the lack of evidence for a population decline, *Litoria ewingii* likely qualifies for the listing of Least Concern under the IUCN Red List criteria (IUCN 2022).

Litoria calliscelis (Peters, 1874)

Suggested common name: South Australian Tree Frog
Figs 11, 12

Hyla calliscelis. Peters, W.C.H. (1874) Über neue Amphibien (*Gymnopis, Siphonops, Polypedates, Rhacophorus, Hyla, Clyclodus, Euprepes, Clemmys*). *Monatsberichte der Königlichen Preussische Akademie des Wissenschaften* zu *Berlin*, 1874, 616–624.

Lectotype. ZMB 92805 (adult male), collected in the vicinity of Adelaide, South Australia, by Richard Moritz Schomburgk, natural historian, botanist, and curator of the Adelaide Botanic Gardens from 1865 until his death in 1891.

Synonyms.

Hyla inguinalis Ahl, 1935. Holotype: ZMB 14080, Type locality: "Südaustralien (wahrscheinlich Umgebung von Adelaide)" = South Australia (probably around Adelaide). Ahl, E. (1935) Beschreibung eines neuen Laubfrosches aus Südaustralien. *Zoologischer Anzeiger,* 109, 252–253.

Material examined. *Hyla inguinalis* and *H. calliscelis* types examined from high-resolution images. For full list of specimens examined in morphometric analyses see Supplementary Table S1 (https://zenodo.org/record/8423599).

FIGURE 11. Lectotype of *Hyla calliscelis*, ZMB 92805, adult male, Adelaide, South Australia. Images provided by Frank Tiller, Museum für Naturkunde, Berlin.

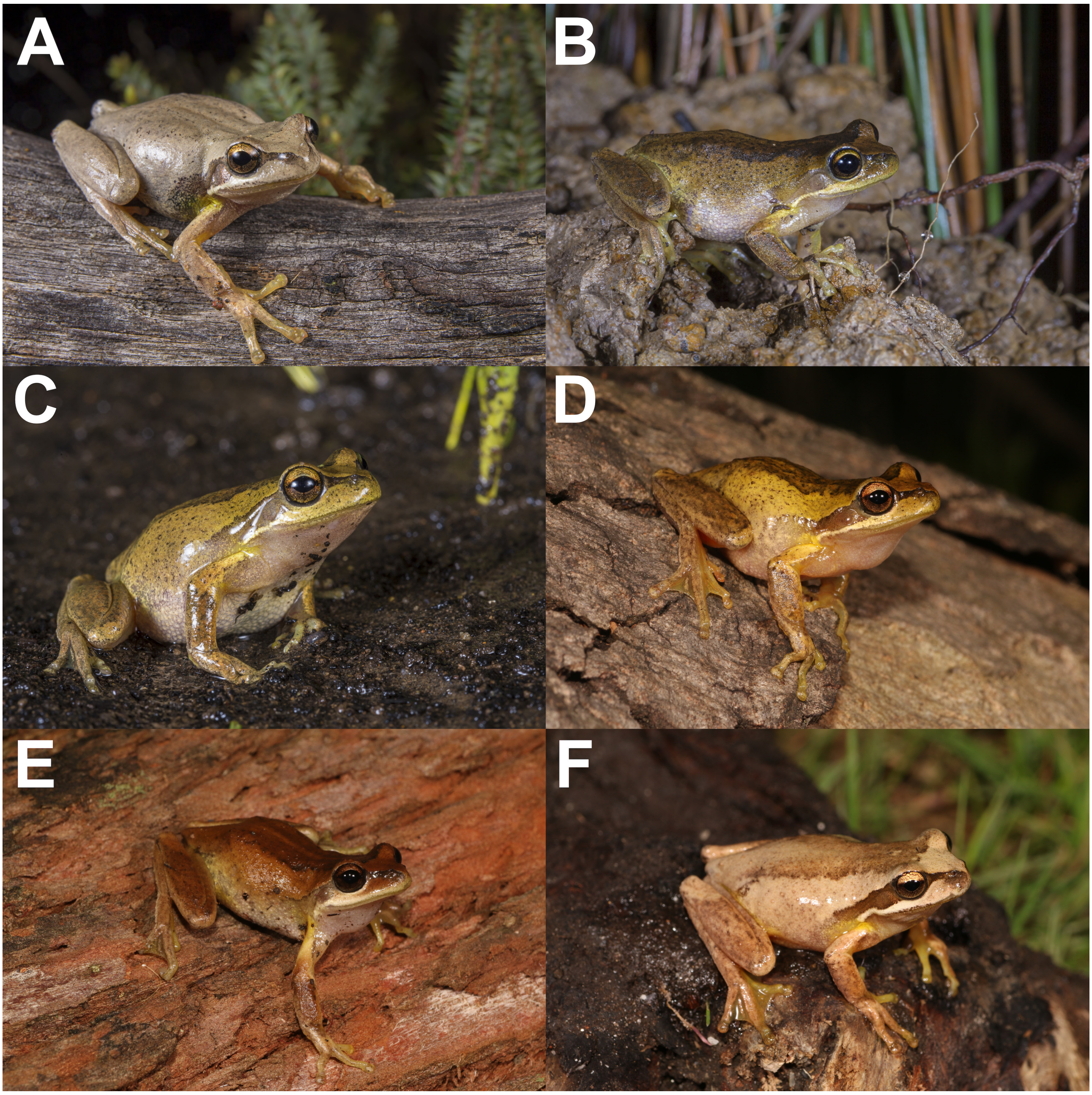

FIGURE 12. Images in life of *Litoria calliscelis*. **A–C)** unvouchered individuals from the Barossa Valley, South Australia (Ryan Francis); **D–F)** unvouchered individuals (Shawn Scott); **D**) Adelaide Hills Region, South Australia; **E**) Adelaide Hills Region, South Australia; **F**) Fleurieu Peninsula, South Australia.

Revised diagnosis. *Litoria calliscelis* is diagnosable from all other members of the *L. ewingii* group by a combination of (1) adult body size of 27–34 mm for males and 28–45 mm for females, (2) moderately robust build, (3) pads wider than fingers (mean Fin3W/Fin3DW = 0.6) and toes (mean Toe4W/Toe4DW = 0.7), (4) webbing on hands vestigial but relatively well-developed on the feet (extending to the 1st subarticular tubercle on the 4th toe [see Fig 8, Type B]), (5) posterior edge of thigh yellow-orange, usually patterned with dark spots or blotches (n=10/11), occasionally plain (n=1/11), (6) usually a single dark spot present in the inguinal region, most often right at the junction of the thigh and body (n=7/11), sometimes 2–3 spots (n=1/11) or plain (n=3/11), (7) genetically by apomorphic nucleotide states at 10 sites in the *ND4* gene (Table 4). Diagnoses of *Litoria calliscelis* and the other species described herein are presented in Table 8 for ease of comparison.

TABLE 8. Diagnoses for the three species of the *Litoria ewingii* Group redescribed or described.

Character	*Litoria ewingii*	*Litoria calliscelis*	*L. sibilus* **sp. nov.**
Adult body size	males 28–35 mm; females 28–45 mm	males 27–34 mm; females 28–45 mm	males 25–34 mm; females 28–43 mm
Build	Moderately robust	Moderately robust	Moderately robust
Finger and toe pad size	Pads wider than fingers (mean Fin3W/Fin3DW = 0.6) and toes (mean Toe4W/Toe4DW = 0.7)	Pads wider than fingers (mean Fin3W/Fin3DW = 0.6) and toes (mean Toe4W/ Toe4DW = 0.7)	Pads wider than fingers (mean Fin3W/Fin3DW = 0.6) and toes (mean Toe4W/Toe4DW = 0.7)
Extent of webbing	Webbing vestigial on the hands, relatively well-developed on the feet (typically extending from 1st–halfway between 1st and 2nd subarticular tubercle on the 4th toe, rarely extending to the 2nd)	Webbing vestigial on the hands, relatively well-developed on the feet (extending to the 1st subarticular tubercle on the 4th toe)	Webbing vestigial on the hands but relatively well-developed on the feet (extending to the 1st subarticular tubercle on the 4th toe)
Posterior thigh patterning	Posterior edge of thigh orange-yellow and usually plain, unpatterned (n=27/32), but sometimes with scattered dark spots, flecks or lines (n=5/32)	Posterior edge of thigh orange-yellow and usually patterned with dark spots or blotches (n=10/11), occasionally plain (n=1/11)	Posterior edge of thigh orange-pink, usually patterned with dark spots and blotches (n= 26/27), occasionally plain (n=1/27),
Inguinal patterning	Dark spots in the inguinal region usually absent (n=29/33), although occasionally present (n=4/33)	Usually a single dark spot present in the inguinal region, most often at the junction of the thigh and body (n=7/11), sometimes 2–3 spots (n=1/11) or plain (n=3/11)	Dark spots or blotches in inguinal region usually absent (n=23/27), sometimes a single spot may be present at the junction of the thigh and body (n=4/27),
Number of sites with apomorphic nucleotide states in the ND4 gene (Table 4)	8	10	15

Description of lectotype. We describe the lectotype based on images of the specimen after more than 148 years in preservative (Fig. 11). Habitus moderately slender. Head slightly longer than wide, widest at commissure of the jaws. Tympanum oblong-shaped, smaller than eye, and partially obscured by a tympanic fold. Snout rounded in dorsal and lateral profiles. Fingers unwebbed and toes with moderate webbing. Finger and toe pads wider than digits. Sub-articular tubercles and metacarpal tubercles visible, inner-metatarsal tubercles prominent, oblong-shaped and approximately half the length of the fourth toe. Darkened nuptial pads present indicating specimen is an adult male. Legs relatively long and slender. Texture of dorsal surface smooth with minor scattered tubercles, ventral surface coarsely granular, inguinal region, lower surface of tibia, upper surface of thighs and throat smooth.

Colour in preservative. Dorsum light pinkish yellow with darker grey longitudinally bifurcated bands from between eyes to vent. Ventral surface creamy yellow. Broad grey facial mask extends from nare through eye to lateral zone, with cream stripe runs from below eye to shoulder. A distinct single dark spot present in the inguinal pocket, at the junction of the thigh and abdomen. Posterior edge of thigh coppery with numerous dark rounded spots.

Variation. Summary of variation in morphometric characters for each sex is presented in Table 6.

Colour and pattern (in life). Variation in colour described from images taken in life (Fig 12). Dorsum base colour varies from cream, grey, coppery to golden, with dark burnt brown longitudinally aligned and bifurcated (occasionally completely separated) bands extending from between eyes to vent. Upper surface of legs cream grey to coppery green or gold. Posterior edge of thigh yellow-orange, usually patterned with dark spots or blotches (n=10/11), occasionally plain (n=1/11). Distinctive golden patch on posterior edge of shoulder and armpit. Rose-gold, pink to burnt-brown stripe runs from rostrum through eye and fades into the lateral zone. Pale to gold cream stripe runs from below eye to tympanum or shoulder. Iris copper-gold.

Advertisement Call. Call description is based on the calls of 59 individuals. The advertisement call of *Litoria calliscelis* has a duration of 1.57–5.66 s (mean 3.35 s) comprising 7–32 pulsed notes (mean 16) with a short duration 0.09–0.23 s (mean 0.16 s). Typically calls have the shortest note at the beginning (79.7%) with the longest note at the end (71.2%) (Fig. 7). Amplitude rises and falls throughout the length of a note and the call but with no distinctive pattern. Dominant frequency ranges between 2063–2809 Hz (mean 2444 Hz).

Comparison with other species. *Litoria calliscelis* does not occur in sympatry with any other species in the *Litoria ewingii* Group making identification straightforward within its natural range. Where collection location is unknown though, *Litoria calliscelis* may be distinguished from *L. ewingii* by a thigh pattern usually comprising dark spots and blotches (versus typically plain in *L. ewingii)* and somewhat reliably from *L. sibilus* by having an inguinal pattern consisting of a single dark spot in the inguinal pocket at the juncture of the thigh and abdomen (versus usually absent in *L. sibilus*, although faded spots are present in a small number of specimens (n=4/27).

Distribution and habitat. Endemic to the Southern Flinders ranges, Mount Lofty Ranges, Fleurieu Peninsula, Adelaide coastal Plain, and lower Murray River valley. Extends as far north as Port Augusta, with the eastern limit of its range bounded by the Murray River, extending from Lake Albert and Lake Alexandrina upstream to at least Blanchetown. Absent from the Yorke and Eyre Peninsulas, although three outlying observational records in the ALA dataset from Port Lincoln may require further validation.

Occurs in a variety of habitats including forests, heathlands and agricultural and suburban areas.

Ecology and reproduction. The species is recorded relatively commonly via FrogID (>1,600 records from 10 November 2017–30 June 2022), and is often heard calling in disturbed areas, with 39% of FrogID records of the species documented as being in urban habitats and 37% of records in rural areas. Males occur year-round in association with rainfall, with a distinct peak during June–October. Breeding occurs in static permanent and ephemeral waterbodies, such as dams, ponds, swamps, inundated ditches and streamside ponds and pools (Anstis 2017; S. Scott *pers. comm.).* For a detailed description of tadpole development and morphology, see Anstis (2017).

Conservation status. AOO and EOO for *Litoria calliscelis* were calculated at 2852 km^2 and 37,649 km^2 respectively. Based on these calculations, a high relative abundance in FrogID submissions, and a lack of evidence for a population decline or obvious fragmentation, *Litoria calliscelis* likely qualifies for listing as Least Concern under the IUCN guidelines (IUCN 2022).

Litoria sibilus **sp. nov.**

Suggested common name: Kangaroo Island Tree Frog
Figs 13, 14

Holotype. SAMA R37403 (tissue voucher ABTC33479), adult female, 1.5 km NW Rocky River, Flinders Chase National Park, Kangaroo Island, South Australia (35.94ºS, 136.73ºE), Collected by David Armstrong on 12th November 1990.

Material examined. See Supplementary Table S1 (https://zenodo.org/record/8423599) for details of all material examined.

Holotype measurements (mm). SVL 39.4, FOL 29.7, TL 20.3, THL 20.0, HW 11.8, IOD 3.9, DFE 6.9, IND 3.2, NS 2.4, EN 3.0, ED 4.2, HDD 5.1, SL 4.9, HL 17.0, UAL 6.9, LAL 9.2, HAL 12.2, AL 20.2, FL 17.44, IMT 1.2, TMP 2.25, Fin3DW 1.69, Fin3W 1.05, Toe4DW 1.4, Toe4W 0.89.

Diagnosis. *Litoria sibilus* is diagnosable from all other members of the *L. ewingii* group by a combination of (1) adult body size 25–34 mm for males and 28–43 mm for females, (2) moderately robust build, (3) pads wider than fingers (mean Fin3W/Fin3DW = 0.6) and toes (mean Toe4W/Toe4DW = 0.7), (4) webbing on hands vestigial but relatively well-developed on the feet (extending to the 1st subarticular tubercle on the 4th toe [see Fig 8, Type B]), (5) posterior edge of thigh orange-pink, usually patterned with dark spots and blotches (n= 26/27), occasionally plain (n=1/27), (6) dark spots or blotches in inguinal region usually absent (n=23/27), sometimes a single spot may be present right at the junction of the thigh and body (n=4/27), (7) genetically by apomorphic nucleotide states at 15 sites in the *ND4* gene. Diagnoses of *Litoria sibilus* and the other species described herein are presented in Table 8 for ease of comparison.

FIGURE 13. Holotype of *Litoria sibilus* **sp. nov.**, SAMA R.37403, adult female, Flinders Chase National Park, Kangaroo Island, South Australia.

Description of holotype. Habitus moderately slender. Head longer than wide (HW/HL = 0.7), tympanum oblong-shaped, smaller than eye (TMP/ED = 0.5), and partially obscured by a tympanic fold. Snout rounded in dorsal and lateral profiles. Fingers and toes slender, fingers unwebbed and toes with moderate webbing. Finger and toe pads wider than digits (Fin3W/Fin3DW and Toe4W/Toe4DW = 0.6). Sub-articular tubercles and metacarpal tubercles visible, inner-metatarsal tubercles prominent, oblong-shaped and approximately two-thirds the length of the fourth toe. Oviducal follicles visible from abdominal cavity indicates specimen is a gravid female. Arms and legs moderately long (TL/SVL = 0.5) and slender. Texture of dorsal surface smooth, ventral surface coarsely granular.

Colour in preservative. Dorsum uniformly silver-grey. Yellow-green grey ventral surface. A single faint dark spot present in the inguinal pocket, at the junction of the thigh and abdomen. Subtle pink posterior edge of thigh with numerous dark rounded spots. White stripe present from below eye to shoulder and above eye to upper tympanum.

Variation. Summary of variation in morphometric characters for each sex is presented in Table 6.

Colour and pattern (in life). Variation in colour described from images taken in life (Fig 14). Dorsum base light silvery grey to copper, with dark burnt brown, black-edged, longitudinally aligned, and bifurcated bands extending from between eyes to vent. Upper surface of legs coppery pink, posterior edge of thigh orange-pink usually patterned with dark spots and blotches (n= 26/27), occasionally plain (n=1/27). Dark spots or blotches in inguinal region usually absent (n=23/27), sometimes a single spot may be present right at the junction of the thigh and body (n=4/27). Silver, pink, grey to copper stripe extends from rostrum through eye to lateral zone. White stripe present from below eye to shoulder. Iris gold.

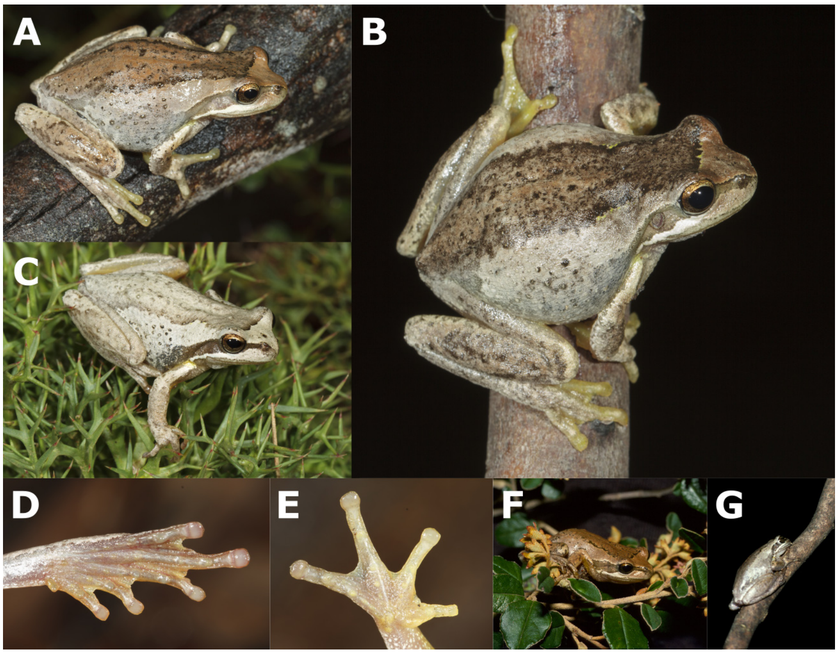

FIGURE 14. Images in life of *Litoria sibilus* **sp. nov.** **A–E** unvouchered individuals (Mark Sanders). **A**) Parndana, central Kangaroo Island; **B**) Parndana, central Kangaroo Island, **C**) Vivonne Bay, southern Kangaroo Island; **D**) hindfoot of individual in A; **E**) hand of individual A; **F–G** unvouchered individuals, Kangaroo Island (Department of Environment and Water, South Australian Government).

Etymology. The specific epithet, *sibilus*, is a masculine Latin 2nd declension noun meaning whistle or hiss. It is used in apposition to the genus name.

Advertisement Call. Call description is based on the calls of six individuals. The advertisement call of *Litoria sibilus* has a duration of 1.48–2.34 s (mean 1.78 s) and comprises 8–11 (mean 9) pulsed notes. Notes are typically short 0.1–0.28 s (mean 0.17 s) with the shortest note at the beginning (83.3% of calls) and the longest note at the end (50%). Notes have limited intervals between them and are not distinctly pulsed. In 50% of calls, amplitude rose to a peak in roughly the middle of each note before descending, giving a tent-like appearance (Fig. 7). Dominant frequency had a short range of 2156–2541 Hz (mean 2451 Hz).

Comparison with other species. *Litoria sibilus* is isolated from all other members of the *L. ewingii* group, separated from the closest species, *L. calliscelis*, across a 13 km stretch of sea known as the Backstairs Passage, which separates Kangaroo Island from the adjacent mainland. For a description of colour/pattern features potentially useful for identification where collection locality is unknown, see *Litoria ewingii* and *L. calliscelis* above.

Distribution and habitat. Endemic and widespread on Kangaroo Island (4,405 sq km), Australia's third largest island, situated off the South Australian coast.

Habitat poorly known but has been recorded in forest, heathland and disturbed agricultural land.

Ecology and reproduction. The species is uncommonly recorded via FrogID (68 records from 10 November 2017–30 June 2022), but is often heard calling in disturbed areas, with 6% of FrogID records of the species documented as being in urban habitats and 74% of records in rural areas. Breeding period appears to be extended, with males recorded calling in January–November, with the majority of FrogID submissions from between March–May. Breeding habitat poorly known but appears to include static ephemeral and permanent waterbodies such as dams, ponds, swamps, inundated ditches and streamside ponds and pools. For a detailed description of tadpole development and morphology specific to this taxon, see Anstis (2017).

Conservation status. *Litoria sibilus* has the most restricted distribution of all species within the *Litoria ewingii* Group. AOO was calculated for the species at 268 km^2 and EOO 3,743 km^2. Under criterion B of the IUCN Red List guidelines, these calculations potentially qualify the taxon for Endangered listing (AOO <500 km^2; EOO

<5,000km^2). However, given there is a lack of baseline information indicating severe fragmentation, decline or fluctuation in population size, we suggest a conservative listing of Data Deficient to be appropriate for this taxon until further information becomes available. Of particular concern is the potential threat posed by the devastating bushfires that occurred on Kangaroo Island in 2019–2020, which burned through 45.9% the island's landmass (Bonney *et al.* 2020). Targeted surveys of *Litoria sibilus* are required to establish a population baseline and assess post-fire population health and recovery.

Discussion

Our study recognises two additional species in the *Litoria ewingii* Group, bringing the total number of species in the group to nine. We found the species to be allopatric due to landscape and marine barriers, with no evidence of contemporary gene flow and a depth of genetic structuring that implies ancient speciation with the latter supported by the co-location of the phylogenetic breaks coinciding with well-known biogeographic barriers—the Murray River Basin and Backstairs Passage, a stretch of sea separating Kangaroo Island from mainland South Australia.

The level of genetic divergence observed between *Litoria sibilus* and its mainland counterparts suggests a vicariance event that pre-dates the last terrestrial interconnections between Kangaroo Island and mainland Australia, which are estimated to have occurred at least twice in the past 500 Kya (Dissanayake *et al.* 2022; Dubey & Shine 2010; Symula *et al.* 2008). Similar diversification patterns have been observed for other Kangaroo Island vertebrates, including the frog, *Crinia signifera* (Symula *et al.* 2008), and skink, *Acritoscincus duperreyi* (Dissanayake *et al.* 2022; Dubey & Shine 2010), with divergence estimates for populations of both taxa predicted to have occurred in the Plio-Pleistocene, far pre-dating the most recent land bridges. This suggests the presence of an additional barrier to dispersal that prevented some organisms from traversing the Backstairs Passage land bridge during more recent periods of lowered sea-level. Such barriers might have included sub-optimal intervening habitat across the land bridge or interspecific limitations in dispersal capability between species. Interestingly, the level of divergence we observed for Tasmanian populations of *Litoria ewingii* from mainland populations is much shallower than for Kangaroo Island populations, despite both having a similar history of terrestrial connections with the mainland. This could either indicate that *Litoria ewingii* has a greater dispersal capacity than its congeners, or that the habitat across the Bass Strait land bridge was more conducive to dispersal, allowing episodic exchange of genes between populations across the land bridge during more recent periods of connection with the mainland.

Our study also highlights the significance of the Murray River Basin as a biogeographic barrier in southern Australia, reflecting the patterns of Plio-Pleistocene phylogenetic structuring observed for several other organisms across the region, including the frog: *Crinia signifera* (Symula *et al.* 2008), skinks: *Acritoscincus duperreyi* (Dubey & Shine 2010, Dissanayake *et al.* 2022), *Tiliqua rugosa* (Ansari *et al.* 2018) and *Lampropholis guichenoti* (Chapple *et al.* 2011), marsupial dunnart: *Sminthopsis crassicaudata* (Cooper *et al.* 2000) and grasshoppers: *Vandiemenella viatica* (Kawakami *et al.* 2009). The Murray River Basin is characterised as a widespread low-lying depression which has been subject to repeated marine transgressions dating back to mid-late Miocene (Ansari *et al.* 2018, Chapple *et al.* 2011). Tectonic activity in the region ~3–4 Mya resulted in damming of the Murray River and subsequent major expansion of surface waters, leading to the establishment of Lake Bungunnia, a paleo mega-lake covering approximately 50,000 km^2, which persisted until ~700Kya (Ansari *et al.* 2018, Byrne *et al*. 2008, Dissanayake *et al.* 2022). The physical dispersal barrier presented by Lake Bungunnia, coupled with the onset of a phase of dramatic habitat change associated with global cooling and aridification commencing in the late Pliocene, is proposed to have fragmented many formerly continuous populations into discrete refugia (Ansari *et al.* 2018, Chapple *et al.* 2011, Dissanayake *et al.* 2022, Dubey & Shine 2010).

The present-day distributions of *Litoria calliscelis* and *L. ewingii* remain disjunct over a distance of only 80 km across the Coorong Region and northern Naracoorte Coastal Plain. The habitat in this intervening zone, characterised by saline intertidal mudflats and semi-arid mallee woodland, contains limited fresh surface water for breeding, presenting an effective contemporary barrier to dispersal between the species. Given the taxa described herein are isolated due to allopatry, the question remains whether they have diverged enough to be reproductively incompatible. Genetic incompatibility may arise between diverging species through the gradual accumulation of 'barrier' loci which are resistant to genomic introgression, eventually resulting in pre- or post-zygotic isolation between the species (Dufresnes *et al.* 2021). Given the species described herein have clearly accumulated a high number of fixed allelic differences in geographic isolation (Table 5), it seems plausible that a proportion of these loci may contribute to genetic incompatibility between the taxa. However, of note, Watson & Littlejohn (1978) reported no developmental abnormalities in tadpoles hatched from an *in vitro* cross experiment between a female northern *Litoria ewingii* (Carabost, NSW) and what we presume to be a male *Litoria calliscelis* based on collection locality (Myponga, Fleurieu Peninsula, SA). This is interesting given the relatively high number of fixed allelic differences we found between these taxa (482) when compared to *Litoria ewingii* and *L. paraewingi* (66), which are known to be highly incompatible based on the significant proportion of embryos that develop lethal anopthalmia in hybrid experiments (Watson *et al*. 1971, Watson & Littlejohn 1978). Nevertheless, without further experimental hybridisation data for the taxa described here, these questions remain hypothetical, considering that the *in vitro* crosses effectively addressed only the viability of F_1 hybrids as far as the late larval stage. Due to the presence of strong contemporary barriers to dispersal, natural contact between these species is presently unlikely, but the impact of predicted anthropogenic climate change on habitats in south-eastern South Australia is yet to be explored.

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