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Systematics of the *Litoria citropa* (Anura: Hylidae) Complex in Northern New South Wales and Southern Queensland, Australia, With the Description of a New Species

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ABSTRACT. We describe a new hylid frog, *Litoria daviesae* n.sp., from the highlands of mid-eastern New South Wales, Australia. The new species was formerly included in *L. subglandulosa*, from which it is now distinguished by allozyme and mitochondrial DNA profiles, colour and adult size. The geographic ranges of both species include several widely spaced conservation reserves. The conservation status of *L. subglandulosa* requires further investigation in the light of recent reported declines. *Litoria daviesae* n.sp. occurs in a series of apparently disjunct populations above 400 m altitude along the eastern escarpment and adjacent tablelands of the Great Dividing Range. The larvae of *L. daviesae* n.sp. occur in permanent streams and the adults are closely associated with the riparian zone.

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The forests of the coast and Great Dividing Range of eastern Australia contain a number of endemic hylid frog radiations. One of these, the *Litoria citropa* species group (Tyler & Davies, 1978, 1985), presently comprises a number of smaller leaf green coloured species and two larger species, *L. citropa* and *L. subglandulosa* both with prominent submandibular glands. Except for a nomenclatural issue concerning the usage of a species epithet (Tyler & Anstis, 1983), the systematics of the two larger species has been stable since *L. subglandulosa* was described by Tyler & Anstis (1975). The reproductive and larval biology and distributions of the species are well described (Anstis & Littlejohn, 1996).

Several species of frogs from eastern Australia have either disappeared or have suffered notable declines in their abundance and range (Ingram & McDonald, 1993; Richards

et al., 1993; Mahony, 1996). In particular, two species of hylids, *Litoria castanea* and *L. piperata*, originally found within the range of *L. subglandulosa*, have disappeared while *L. subglandulosa* has undergone a decline in abundance (Mahony, 1996). Historically, *L. subglandulosa* was known from a small number of locations, and its range has been revised recently to include the northern catchment of the Hunter River in central New South Wales (Anstis & Littlejohn, 1996).

Litoria subglandulosa was listed as vulnerable in the New South Wales Threatened Species Conservation Act, 1995, because it was considered to be a habitat specialist, with a restricted distribution and Lunney *et al*. (1996a,b) presented evidence of population declines. The species has never been considered to be common (Tyler & Anstis, 1975; Anstis & Littlejohn, 1996; Anstis, 1997). Recent comprehensive surveys show that it has declined in abundance from particular geographic regions (NSW NPWS 1994; Anstis, 1997).

Recent molecular and morphological analyses of the species groups of hylid frogs from eastern Australia have identified the presence of additional and in some cases "cryptic" species (Donnellan *et al*., 1999). As part of the assessment of the conservation status of *Litoria subglandulosa*, we undertook a molecular and morphological analysis to assess whether the species harboured any cryptic taxa. The presence of undetected taxa within what is thought to be single taxon that is under investigation for conservation and management can potentially lead to mismanagement of the conservation of biodiversity (Parnaby, 1991; Donnellan *et al*., 1993). We have also made an assessment of the conservation status of populations based on specimen records from museum collections, biological survey data, and the field notes of individual workers.

Materials and methods

Material examined. Specimens examined are listed in the Appendix. Institutional abbreviations follow Leviton *et al*. (1985). We used a number of other species of *Litoria* as outgroups or for other comparative purposes. We included taxa from the *L. phyllochroa* species complex, which appears to comprise more species than are presently recognized (Donnellan *et al*., 1999). These are labelled *L. nudidigitus, L. phyllochroa, L. pearsoniana*-1 and *L. pearsoniana*-2.

Allozyme electrophoresis. Frozen tissues were available from 24 specimens of *Litoria* from nine localities in northern New South Wales (Appendix, Fig. 1). These included *L. citropa, L. pearsoniana*-2 and *L. spenceri* to allow an assessment of evolutionary relationships among the taxa of the *L. citropa* species group and to help identify misidentified specimens that might be present in the sample. Allozyme electrophoresis of liver homogenates was performed on Cellogel (Chemetron, Milan) according to the methods of Richardson *et al*. (1986). The enzyme products of 32 presumptive loci were scored. The enzymes (and proteins) stained and Enzyme Commission numbers were: aspartate aminotransferase (AAT, EC 2.6.1.1), aconitate hydratase (ACOH, EC 4.2.1.3), aminoacylase (ACYC, EC 3.5.1.14), adenylate kinase (AK, EC 2.7.4.3), dihydrolipoamide dehydrogenase (DDH, EC 1.8.1.4), enolase (ENO, EC 4.2.1.11), fructose-bisphosphatase (FBP, EC 3.1.3.11), fumarate hydratase (FUMH, EC 4.2.1.2), glycerol-3-phosphate dehydrogenase (G3PDH, EC 1.1.1.8), glucose-6-phosphate isomerase (GPI, EC 5.3.1.9), glutamate dehydrogenase (GTDH, EC 1.4.1.3), 3-hydroxybutyrate dehydrogenase (HBDH, EC 1.1.1.30), isocitrate dehydrogenase (IDH, EC 1.1.1.42), cytosol aminopeptidase (LAP, EC 3.4.11.1), L-lactate dehydrogenase (LDH, EC 1.1.1.27), lactoylglutathione lyase (LGL, EC 4.4.1.5), malate dehydrogenase (MDH, EC 1.1.1.37), mannose-6-phosphate isomerase (MPI, EC 5.3.1.8), nucleoside-diphosphate kinase (NDPK, EC 2.7.4.6), dipeptidase (PEP-A, EC 3.4.13.?), tripeptide aminopeptidase (PEP-B, EC 3.4.11.?), proline dipeptidase (PEP-D, EC 3.4.13.?), phosphoglycerate mutase (PGAM, EC 5.4.2.1), phosphogluconate dehydrogenase (PGDH, EC 1.1.1.44), phosphoglycerate kinase (PGK, EC 2.7.2.3), phosphoglucomutase (PGM, EC 5.4.2.2), and triose-phosphate isomerase (TPI, EC 5.3.1.1). Alleles were identified by comparison with samples that were repeatedly included on each gel (internal controls) and through critical side-by-side comparisons (line-ups; see Richardson *et al*., 1986). To facilitate the analysis of data, specimens of a single genetic type from a single location (i.e., where there were no fixed differences [Richardson *et al*., 1986]), were treated as an Operational Taxonomic Unit (OTU) (Tables 1 and 2, Fig. 1). Evolutionary relationships among the OTUs were recovered by conducting heuristic searches under the maximum parsimony criterion of optimality (MP) implemented in PAUP* 4.0b3 (Swofford, 1999). Loci were treated as characters, alleles as unordered character states and polymorphisms as uncertainties. Robustness of phylogenetic hypotheses was estimated from bootstrap proportions among 10,000 pseudoreplicates with the "fast" heuristic search option in PAUP* 4.0b3. A genetic distance analysis was based on Cavalli-Sforza chord distances between OTUs (Cavalli-Sforza & Edwards, 1967) generated with BIOSYS-1 (Swofford & Selander, 1981) using the tree-building Neighbor Joining (NJ) algorithm implemented with the NEIGHBOR routine in PHYLIP Version 3.5 (Felsenstein, 1993).

Mitochondrial DNA. See the Appendix for details of specimens examined. Liver samples were kept frozen at -70°C until used for DNA extraction. Total cellular DNA was extracted with the salting out procedure of Miller *et al*. (1988) and stored at -20°C. For polymerase chain reaction (PCR) amplifications, 50–100ng of DNA was added to a 50µl reaction mixture containing 4mM $MgCl_2$, 1X reaction buffer, 0.8mM dNTPs 0.4mM primers, 1 unit of Biotech *Tth* plus DNA polymerase and the remaining volume of dH_2O. Reaction mixtures were overlayed with 60–90µl of mineral oil. The primers used for *16S rRNA* amplification (16sar and 16sbr) were designed by Cunningham *et al*. (1992), and for *ND4* (Limno2 and ND4) were designed by C. Schauble, University of Queensland and Arévalo *et al*. (1994) respectively. Amplification was carried out on a Corbett FTS-320 Thermal Sequencer and comprised a single cycle of denaturation for 3 min at 94°C, annealing for 45 s at 55°C and extension for 1 min at 72°C, followed by 29

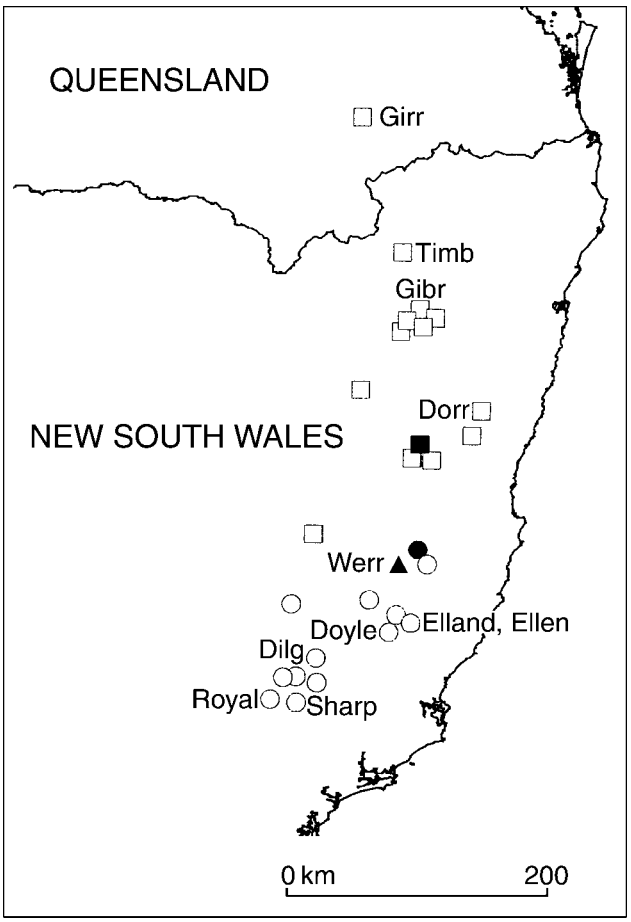

Figure 1. Map of eastern Australia showing collection locations for specimens of *Litoria* examined. Abbreviations refer to site locations where specimens were examined by molecular methods (see Appendix for key to codes). *Litoria subglandulosa* (□), *L. daviesae* (○), specimens of undetermined species (●), and type localities of *L. daviesae* (▲) and *L. subglandulosa* (■).

cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 1 min, ending with a single extension step of 72°C for 6 min. PCR products were purified for sequencing using a Bresa-Clean DNA Purification Kit (Bresatec), following the manufacturer's protocol for DNA extraction from solutions.

Each sample had both strands sequenced directly from the PCR product using the original PCR primers. Products were cycle-sequenced on a Corbett FTS-1 Thermal Sequencer using the Applied Biosystems PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit, following the manufacturer's instructions. The sequencing program consisted of 25 cycles of 94°C for 30 s, 50°C for 15 s and 60°C for 4 min. Cycle-sequenced product was electrophoresed and viewed on an Applied Biosystems Model 373A Sequencing System.

Partial sequences of *16S rRNA* from *L. nudidigitus, L. phyllochroa, L. pearsoniana*-1 and -2, and *L. spenceri* were obtained from Donnellan *et al.* (1999) and are available from the European Bioinformatics Institute Server: www address: ftp://ftp.embl-heidelberg.de/pub/databases/embl/align/ (alignment ds38337). *Litoria caerulea* was used as an outgroup, as an ongoing study of relationships within the Australasian Hylidae shows this species to be a member of one of several species groups closely related to the *L. citropa* species group (Donnellan & Monis, unpubl. data). Sequences were aligned with CLUSTALW (Thompson *et al.*, 1994) and improved by eye without reference to secondary structure models as the alignment required the insertion of few gaps. GenBank accession numbers for these sequences are *16S rRNA*: AF2822609–14, *ND4*: AF282598–608. Evolutionary trees, constructed with the maximum parsimony (MP) or maximum likelihood (ML) criteria of optimality, were found with heuristic searches implemented in PAUP* 4.0b3 (Swofford, 1999). The robustness of phylogenetic hypotheses was tested with bootstrapping.

Morphological analysis. Measurements were taken with vernier callipers to the nearest 0.5 mm from formalin-fixed, alcohol-preserved specimens after the method of Tyler (1968). Measurements taken were: snout-vent length (SV), head length (HL), head width (HW), eye to naris distance (EN), internarial span (IN), eye length (E), greatest length of tympanum (T), eye to tympanum (ET), interocular span (IO), arm length (A), longest finger (4th) length (F), tibia length (TL), and longest toe (4th) length (P). T and ET were measured under a dissecting microscope. Sex was determined by the presence or absence of a nuptial pad in adults or by examination of gonad morphology after dissection

Discriminant function analysis (DFA) was performed on log-transformed measurement data. Only individual specimens that had been given an *a priori* group classification based either on their genotype (allozyme profile or mitochondrial haplotype) or distribution (see below) were used to derive the DFA equation. All other measured specimens were then ascribed to one of the known OTUs using the DFA. Statistical analyses were conducted with the program Statistica release 5.1, 1997 edition (StatSoft, 1997).

Assessment of historical and current distribution. Distributional records were obtained from the Australian (AMS), Queensland (QM) and South Australian (SAMA) museums, literature, and extensive field surveys in northeastern New South Wales conducted over the past seven years. Targeted systematic searches were conducted where suitable habitat occurred. Stream transects were conducted by day and night for a distance of 500 m. At the middle and ends of a transect, a sequence of male calls was broadcast for five minutes, at a level slightly greater than that normally produced by males, to elicit response from any males that may be present but not calling.

Results

Allozyme electrophoresis. Allele frequencies at the 32 loci resolved among the 12 OTUs are presented in Table 1. These data were converted into a matrix of percentage fixed allelic differences among the OTUs. A strict consensus tree summarising the 176 trees of length 35 steps found with MP is presented in Fig. 2a. Two lineages are apparent among the 8 OTUs classified *a priori* as *L. subglandulosa*: group 1 includes OTUs: Dilg, Elands, Ellen, Royal, Werr, and Doyle; and group 2 includes OTUs: Dorr, Gibr, and Timb. The groups differ by fixed allelic differences at four loci: *Acoh-2, Acyc, Idh-1,* and *Mpi* (Table 1) and are genetically uniform within each group with no fixed differences between OTUs within group 2. An apparent fixed difference at *Aat-2* in group 1 is due most likely to the small sample size. Each group is genetically well differentiated (approximately 50% fixed allelic differences) from the other members of the *L. citropa* species group included and the monotypic *L. spenceri* species group. The phenotype of individual SAMA R51060, typed for the four diagnostic loci only, was *Acoh-2*^{c}, *Acyc*^{b}, *Idh-1*^{ac}, and *Mpi*^{e}. This specimen is assignable to group 1 on the genotypes at these markers. Groups 1 and 2 are defined by apomorphic character states, group 1 at *Acyc*^{a} and *Idh-1*^{c} and group 2 at *Mpi*^{c}. The *Acoh-2* locus was parsimony uninformative. In the NJ tree generated from the genetic distance analysis (not shown), groups 1 and 2 were also present.

Mitochondrial DNA. A total of 547 aligned sites from the *16S rRNA* nucleotide sequences were available for analysis from the 27 individuals sequenced. A total of 461 sites were invariant, 86 were variable and 46 were parsimony informative, the variable sites are listed in Table 2. Eleven different haplotypes were observed. Two haplotypes differing by 1.1% sequence divergence were found in *Litoria citropa*, a single haplotype was found among the 13 group 1 individuals and two different haplotypes were observed among the six group 2 individuals (haplotype 1: SAMA R51052; haplotype 2: SAMA R39108, R51051, ABTC 68396–8). The percentage uncorrected sequence divergence between the two haplotypes observed within group 2 was 0.6% and between these and the group 1 haplotype ranged from 2.1–2.3%. Percentage uncorrected sequence divergence of the outgroup to the 10 ingroup sequences ranged from 4.4–11.1%.

To analyse the phylogenetic relationships among the 11 haplotypes found among the *16S rRNA* sequences, a 645 bp portion of the *ND4* gene was sequenced for each haplotype and the sequences of both genes were combined into a single analysis. The Incongruence Length Difference test of Farris *et al*. (1994), implemented in PAUP* as the "partition homogeneity test", indicated that the sequence partitions could be combined, P = 0.71. The *ND4* sequences contributed 262 variable sites and 188 parsimony informative sites. Ingroup uncorrected sequence divergences for the *ND4* sequences ranged from 1.4–21.7%. A single MP tree, length 694 steps, was found with an unweighted heuristic search (Fig. 2b). Three major lineages are apparent, *L. citropa/spenceri, L. nudidigitus /*

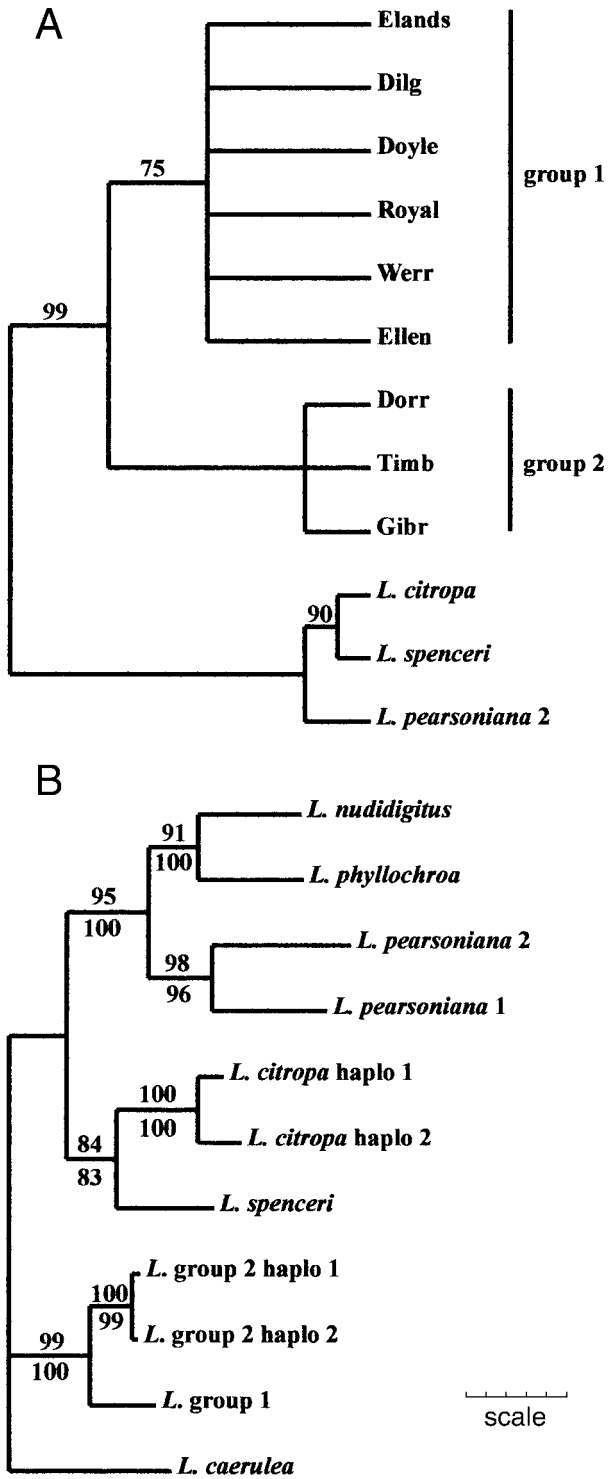

Figure 2. Evolutionary trees of *Litoria* based on (A) a strict consensus tree summarising the 176 equally most parsimonious trees based on the allozyme data. Bootstrap proportions are to the left of relevant nodes, and (B) a MP tree of relationships among concatenated mitochondrial *16S rRNA* and *ND4* nucleotide sequences. Values above and below the nodes represent bootstrap proportions greater than 50% among 2000 MP and 100 ML pseudoreplicates respectively. Scale bar represents 50 steps in MP analysis.

Table 1. Allele frequencies, expressed as a percentage, in 12 OTUs of *Litoria* at 32 loci. Alleles are designated alphabetically, with "a" being the least anodally migrating allele. Where enzymes are encoded by more than one locus, the loci are designated numerically in order of increasing electrophoretic mobility. Where the allele frequencies are not given, the frequency is 100. See the Appendix for an explanation of the OTU codes. The number of individuals sampled from each OTU is given at the head of each column, except when fewer individuals were successfully typed. In the latter case sample sizes are indicated by the number in superscript beside the first allelic frequency entry for a locus. The following loci were invariant: *Gpdh, Gtdh, Mdh-1, Mdh-2* and *Pgam*.

locus	Dilg	Eland	Ellen	Werr	Royal	Doyle	Dorr	Gibr	Timb	*pear*-2	*citro*	*spenceri*
	3	1	1	3	3	2	1	1	1	1	2	4
Aat-1	b	b	b	b	b[2]	b	b	b	b	b	c	b(67)[3] a(33)
Aat-2	b(83) a(17)	b(50) a(50)	a	b	b(75) a(25)	b(75) a(25)	b	b	b	c	c	c
Acoh-1	b	—	b	b	b	b[1]	b	b	b	a	a	a
Acoh-2	c	c	c	c	c	c	b	b	b	a	a	a
Acyc	a	a	a	a	a	a	c	c	c	—	b	b
Ak	c(17) b(83)	—	b	c(17) b(83)	b	c(25) b(75)	b	b	b	c(50) b(50)	b(75) a(25)	b(87) a(13)
Ddh	a	a	a	a	a	a	a	a	a	a	a	b
Eno	b	b	b	b	b[2]	b	b	b	b	b	b	a
Fbp	c(33) b(67)	b	b	c(33) b(67)	b	c(25) b(75)	b	b	b	b	a	b
Fumh	c	c	c	c	c	c	c	c	c	b	b	a
Gpi	a	a	a	a	a	a	b(50) a(50)	a	a	c	c	b
Idh-1	c	—	c	c	c[2]	c	b	b	b	d(50) b(50)	b	b(67)[3] a(33)
Idh-2	d(33) b(67)	b	b	b(83) a(17)	d(50) b(50)	b(75) a(25)	b	b	b	e(50) d(50)	c	c
Lap	b	b	b	b	b	b	b	b	b	a	b	c(12) b(88)
Ldh-1	b	b	b	b	b	b	b	b	b	a	c	a
Ldh-2	b(83) a(17)	b(50) a(50)	b	b(50) a(50)	b	b	b	b	b	b	b	b
Lgl	a	a	a	a	a	a	a	a	a	b	a	a
Mpi	e	e	e	e	e	e	c	c	c	d	e(75) a(25)	b
Ndpk	e(17) d(83)	d	e(50) d(50)	e(83) d(17)	e(33) d(67)	e(50) d(50)	d	d(50) b(50)	d	c	d(50) a(50)	d
PepA	a	a	a	a	a	a	a	—	a	a	b	b
PepB	c	c	c	c	c	c	c	c	c	c	b	a
PepD1	c(50)[1] a(50)	—	a	a	a[2]	a	a	a	a	c(50) a(50)	d(50) b(50)	c(12) b(75) a(13)
PepD2	e	—	f(50) e(50)	e	e	e	e(50) d(50)	e	e	d(50) b(50)	d	c(37) a(63)
Pgdh	b	b	b	b	b[2]	b	b	b	b	a	a	a
Pgk	b	b	b	b	b	b	b	b	b	b	a	a
Pgm	d(17) b(83)	b	b	b(83) a(17)	d(25)[2] b(75)	b	b	b	b	c	c(75) a(25)	c(62) b(25) a(13)
Tpi	a	a	a	a	a	a	a	a	a	b	a	a

phyllochroa/pearsoniana-1 and -2, and group1/group2. The first two lineages form a sister group. The same three major lineages were present in the ML tree found with a heuristic search using the HKY85 model of sequence evolution (not shown). However the *nudidigitus/phyllochroa/pearsoniana*-1 and -2, and group1/group2 lineages form a sister group in the ML tree. Bootstrap proportions from 2000 MP and 100 ML pseudoreplicates (Fig. 2b) show strong support for each of the three major lineages, ie ≥80%, but support is absent for either of the sets of relationships among these lineages apparent in the MP or ML trees. All nodes within each of the three lineages receive strong support from both MP and ML bootstraps, ie >95%. Of particular importance is the reciprocal monophyly of the group 1 and group 2 haplotypes, with very strong support from bootstrapping, ie 99–100%.

Morphological analysis. The *a priori* classified test base comprised specimens identified from either their genotype or on the basis of their distribution. For the non-genotyped sample, specimens from all locations south of 31°14'S were included in group 1 while those from locations north of 30°10'S were included group 2. These dividing lines were determined by allozyme electrophoresis. Specimens from locations between 30°10'S and 31°14'S were entered as *a priori* unclassified cases. Sample sizes were: group 1 ♂♂ n = 19; ♀♀ n = 2; group 2 ♂♂ n = 13; ♀♀ n = 0.

A standard DFA of males only, produced 100% *a posteriori* correct classification of group 1 individuals (n = 19) and 93% correct classification of group 2 individuals (n = 13), with a single individual (AMS R96910 from Gibraltar Range NP) misclassified. A forward stepwise DFA retained seven variables (A, T, IN, HL, ET, TL, E in decreasing order of significance) in the model but resulted in two individuals of group 2 being misclassified (AMS R96910 and SAMA R51052). [Standard Wilk's lambda = 0.203, approx. $F_{(13,19)}$ = 5.721 p<0.000; forward stepwise Wilk's lambda = 0.215, approx. $F_{(7,25)}$ = 13.068 p<0.000].

A standard DFA on the 13 variables with both sexes included produced 100% *a posteriori* correct classification of group 1 (n = 21) and 93% *a posteriori* correct classification of group 2 individuals (n = 13), with a single individual (AMS R96910 from Gibraltar Range NP) misclassified (Wilk's lambda 1.227 approximate $F_{(13,21)}$ = 5.510 p < 0.000). In a forward stepwise DFA, eight variables in decreasing order of significance were used in the model: IN, T, TO, HL, ET, TL, FL and E.

In the DFA of *a priori* unclassified specimens from north of 31°14'S and south of 30°10'S, 26 of the 27 males were classified as group 2. The single exception (SAMA R13506) was collected from a location with 24 other group 2 males.

Because the original test database did not include any group 2 females, we assigned females from locations where males were present to the same taxon as those males. In particular, the holotype of *Litoria subglandulosa* (SAMA R13504), a female, was collected along with 24 males of group 2, making it likely that the name *L. subglandulosa* is correctly applied to this group. For the purposes of describing the two groups, *a priori* unclassified females from locations where male(s) had been identified by either allozymes or DFA were classified as the same group as those male(s). However, specimens from locations from which only *a priori* unclassified females were available were not included in the descriptions.

Systematics

The two groups represent distinct evolutionary lineages. Each is defined by apomorphic allozyme character states in at least one locus. Mitochondrial haplotypes from the two groups were reciprocally monophyletic with approximately 2.3% sequence divergence between lineages for the relatively conservative *16S rRNA* gene and 7.4% for the more rapidly evolving *ND4*. The mtDNA divergence together with the allozyme differentiation could indicate a substantial period of evolutionary divergence. Furthermore, each group is clearly distinct in shape as exemplified by the very high percentage correct classification in the DFA in spite of the overlap in adult male size. We regard these groups as separate evolutionary lineages, possibly of long-standing, and therefore distinct species under the evolutionary species concept (Simpson, 1951; Wiley, 1978; Frost & Hillis, 1990). The type locality of *Litoria subglandulosa* is within the geographic range of group 2, hence the name *L. subglandulosa* is applied to this taxon. As there are no other names available (Cogger *et al*., 1983) we describe group 1 as a new species, *L. daviesae*.

Litoria daviesae n.sp.

Type data. HOLOTYPE, AMS R153052, an adult male from Cobcroft's Trail, Werrikimbe National Park, New South Wales, 31°13'30"S 152°10'12"E (Australia 1:25,000 series sheet 9335-IV-S, grid reference 420465444), collected by M. Mahony, R. Knowles and S. Donnellan on 13 November 1993.

Other material examined. See the Appendix for details of other specimens examined. These specimens are not paratypes.

Diagnosis. A member of the *Litoria citropa* species group characterized by the presence of submandibular gland, reddish-orange colouration of inguinal region and posterior surface of the lower limbs. Can be distinguished from all other members of the *L. citropa* species group except *L. citropa* and *L. subglandulosa* by the presence of the prominent supratympanic fold and submandibular gland. Can be distinguished from *L. citropa* by the absence of vocal sac (present in *L. citropa*), hidden tympanum (distinct in *L. citropa*), dorsum either with or without sparsely distributed, small, raised "warts" in *L. daviesae* versus frequent small, raised "warts" with black pigmentation to distal end in *L. citropa*. Can be distinguished from *L. subglandulosa* by a combination of lightly shagreened skin texture in a majority of *L. daviesae* versus smooth skin in *L. subglandulosa*. In life, the colour of *L. daviesae* "ranges from uniform golden brown with scattered darker mottling over the dorsum to specimens with some small areas of green",

Table 2. Variable sites among 11 aligned nucleotide sequences from *Litoria*. A, *16s rRNA* and B, *ND4*.

A — *16s rRNA*

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                    00111111111111222222222222222222222222222222222233333333333333333333333333333444455555
                    22233445566777111122222223333444555555566666777778000011222233344455555778899014922234
                    38912462856234134823457890139169123568901367134563358902014612315701257021712783812375
L. citropa haplo1   CTCCCCACCGGAACATTTCCAGTAACAATAATAAATTCTTAGTTAAATAGCAAACTATACTTGTTAGCTGCTATCATACCCGTCAC
L. citropa haplo2   .C.T............C.............................T.....................................TG.
L. group 1          ...T....A.A..T.....TTACC.AC.CC........C...C.T.....TC.............A..A.........T..TG.
L. group 2 haplo1   ...T....AA...T......TA.C.AC.CC........C........AT.TT....G...........A.............TG.
L. group 2 haplo2   ...T....A....T......TA.C.AC.CC........C........AT.TT................A...........-.TGA
L. spenceri         ...T.T..A...GA.......ACT.AC..........T.C...C......T...TC.............A..........??????
L. nudidigitus      ...TA.T.A..G.T.C.CT.GA..CAT...T....C.TCCTA..G.G..AT..TT...GAAAA...ATC.TCT...C..T??????
L. phyllochroa      ...T..T.A....T.C.CT.GA..CAT..CT.TT...TCATA..C.GC.AT..TT....AC.-...A...TCT..T....??????
L. pearsoniana-1    ...TT.T.A.....T.C....A..TAT..C.C..T.C.C.T..C.GG..AT..TT....T..-AC.A...TC.G...CA.??????
L. pearsoniana-2    ...TT.T.A...G.T......A..CAT..C....CC.TC.T...G.....T..C.C...AC.-.CTA...TA...T....??????
L. caerulea         T.G....TA...GT....G.TACCCACT.C.....C.TC....C.....AT.C....C.......C...A....T......AAT??
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B — *ND4*

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                    00000000000000000000000000000000000000001111111111111111111111111111111122222222222222
                    00001111122233334444555566666777788889999000001122233334445555666667788889990000011122222
                    36792358979036902581478134692368147803690256847039025814703692358170369258145670690256 8
L. citropa haplo1   ACTACACAAAATCATCCAGTAACATATTATCAACCTAAAACACTTAATACCATTTTCCTCGGTACCATACAAAACCGTATATCTAAC
L. citropa haplo2   G...T........C......G...........GT......T..................AA....GCG...GG............
L. group 1          GTC.A.T.....TG..T.A..C...GC...TTC.T.....T.T.C..C..T.C...T..T.T..TT.C.T....TT......TA.G.
L. group 2 haplo1   GTC.G.T.........T.A..C........TTT.T.....T.T...GC..T.C...T..TAT..TT.C.T.G...T......TA...
L. group 2 haplo2   G.C.G.T.........T.A..C........TTT.T.....T.T....C..T.C...T..TAT..TT.C.T.G...T......TA...
L. spenceri         GT..A....T...C..TGA...T..G....TT.TT.....T.T......TT..CC.TGCTAT..T....T?.....A.T....A..T
L. nudidigitus      GTC.A.TG...C..C...A..CT......CTTTT......TGT.C..CGATG....T.CT.ACC..CA.T....T..C.C..TGG..
L. phyllochroa      .T..A.T....CTG..T.A..CT..GA..CTTCTA....GT.T..G.C.AT....CTT..ACCC..CA.T....T.......TAG.T
L. pearsoniana-1    .T..A.TGT.GC...TA.AC.CT....C.CTTGTT...T.TGT.C.....T.CC..T.C..ACT..CA.TG..G.T.C..G..GGG.
L. pearsoniana-2    .T..AGT..G..T.CTA.AC.TTGA...C.TTT.T....GTGTGC..C...G.C.CTTCTATCT.TCA.TG......CGC..TA..T
L. caerulea         C.CTA.TG.C.C.TC...AAC.......CCTC.ATCGC......A.TC.A..C..CA...AC...T.A.T..G.T...GC.CTA...

                    22222222222222222222222233333333333333333333333333333333333333333444444444444444444444
                    33344444555666677788888990000011122333333444455555666677788889999000011112222223334444 5
                    14701369258124736925689170347925878013679025814579367925812470369235812570134690251479 0
L. citropa haplo1   ATAAGCCCTTCCCAATCTAAACCCAAAGCTTAACATTCTTAAAACAGCCTCCCCATAATACACTCCATAAATTTCAACTTATGACTT
L. citropa haplo2   .....T............GG.T..G........T........G...A.................T.....................
L. group 1          .CCC.TTTC...T.T.....T......ATACGTT.C.T.CC...T.ATG..GTA...TCG.C..TT.C.....CT..A.CT.A.T..
L. group 2 haplo1   ..C.ATTTC...T.TC...GT......ATA.GCT...T.CC...T.A.G..ATAG......C..TT.......CT..A.CT.A.T..
L. group 2 haplo2   ..C.ATTTC...T.TC...GT......ATA.TCT...T.CC...T.A.G..ATAG......C..TT.......CT..A.CC.A.T..
L. spenceri         .CT...T...T....C.CG..T.T...A.........T..G.....A.A.TA.A.........CTTG.....CCT....CGCA....
L. nudidigitus      .C.T....CCT....AT..CT......A.A...T.C.TAC.G.....TA.TGTAG..GC..T...TG..........ACCT.ATT.A
L. phyllochroa      .C.T....C.TT..GAT..CTT...G.A....CT...TA..G....ATGCTA.G.C.CCG.TA.TTG.G....C.G.A.CT.A.T.C
L. pearsoniana-1    ..CTATTA....TG.A.?.CTA...GTATA...TGG.TCC.GT.A.ATA..A.A.CGTCC.C.CTT..TGT.A.T..T.CC.A.TC.
L. pearsoniana-2    .C.TATT......G.A...TT.ATG..ATA.GTTGG.TAC.G..TGATA..A.A...CCC...C.T..T.GAAC..GT.CT.A.T..
L. caerulea         G.C.A.TAA?..T...A......T.C.ATAA..T..CTAC.G.CT.A.T....A...GC.T...TT.......C...AAC.CA.T..

                    44444444444444444444445555555555555555555555555555555555555555566666666666666666666666
                    55555666677777888899999000011112223333334444455566667778888999990000001111111222233333
                    34679023814578036902589145703692580123479034680251234069025812478136789023568913470236 9
L. citropa haplo1   ACAATATACATGCATTAAACCCCGTTATGCTAAAACCCCCCATTAATCGCATTCATCCTTTTATAATGGCTACTGCGGGCATAACTAG
L. citropa haplo2   ..T.................T.A.........................T.........C....G..A......A.............
L. group 1          TT..C...TGC.T...C..TT.TA.CGCA.C..G......T.C.....A....T...T.G.C.C...A.T..T.ATAAT..C...C..
L. group 2 haplo1   TT..C.C..C..A...T.....TACCGCA......T.T..T.C.....ATG..T.C.T.G.C.C...A..C...ATAAT..C......
L. group 2 haplo2   TT..C.C..C..A...T.....TACCGCA......T....T.C.....ATG..T.C.T.G.C.C...A..C...ATAAT..C......
L. spenceri         .T.........A....T..T.....C.C..C.G......T.......?.A...T.C.T..AC...GCAA...T.A.CA.......A.A
L. nudidigitus      T.....C.TT..A...TT.TT.TA.C.CA....GGTTTTT.T...T.TA...CTG.TA.CACTC...A..G...AT.ATTT.T.....
L. phyllochroa      G.GG.G..TTC.T.C.C.....T....C.......T.....T.....T....CT.CTG.AGCCC...A..A...AT.ATTT.T.TATA
L. pearsoniana-1    .TG......C..TG.CT.GATGT.CC..AA.....T...T.T.C...T.G.C.T...A..ACT..GCA.........ATT.C...C..
L. pearsoniana-2    .T......TT..A...C..TTGTA.C...A.G.G.....T.C.C.....A.C.T.CTA...CCC.C.A...GGCATAATT.CT.....
L. caerulea         ..C....G.TCAT..C....TA.TCC...T.........T..CCT.CAA...C..C..AC.......A....TCT.AAT..CTG.A.A
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whereas *L. subglandulosa* is “predominantly green” (“southern” versus “northern” localities respectively in Anstis & Littlejohn [1996]); and larger adult size: *L. daviesae* SV ♂♂ 38.7–53 mm, ♀♀ 59–63.4 mm versus *L. subglandulosa* SV ♂♂ 34.5–40.3 mm, ♀♀ 45.8–50.4 mm. An unpaired Student’s *t* test showed a highly significant difference between the means of the SV of ♂♂ (t = 8.42, df = 52, P<0.0001).

Figure 3. An adult male (SAMA R51059) *L. daviesae* n.sp. from the Ellenborough River, New South Wales photographed in life.

Description of holotype. Head approximately as long as broad (HL/HW 1.04), and approximately one third snout to vent length (HL/SV 0.38). Snout prominent, blunt when viewed from above and in profile. Nostrils more lateral than superior, closer to snout than to eye. Distance between eye and naris equal to internarial span (EN/IN 1.08). Canthus rostralis well defined and straight. Eye relatively large, its diameter greater than eye to naris distance. Pupil horizontal when constricted. Tympanum small, indistinct, and oval with long axis tilted towards eye. Tympanum length approximately half eye diameter (T/E 0.45). Well-developed supratympanic fold, glandular in appearance, that partially obscures tympanic region. Vomerine teeth long curved plates directed posteriorly from the front margin of the choanae. Tongue approximately rectangular.

Fingers long, slender, unwebbed. Subarticular and palmar tubercules prominent. Terminal discs expanded, extending beyond lateral extremities of penultimate phalanx. Fingers in order of length 3>4>2>1. Hindlimb length moderate (TL/SV 0.58). Toes in order of length 4>5=3>2>1. Webbing reaches base of second most distal phalanx on toe 4 and penultimate phalanx on other toes. Subarticular tubercules prominent. Small oval inner metatarsal tubercule present. Terminal toe discs slightly expanded, just extending beyond lateral extremities of penultimate phalanx.

Dorsum lightly shagreened. Abdomen, undersurface of thighs, submandibular area and lateral aspect of body mildly granular. There is a broad and prominent gland covered by smooth skin around the margin of the submandibular area. Pectoral fold and vocal sac are absent.

Dimensions of holotype (mm) SV 44.3, HL 15.4, HW 15.1, EN 3.7, IN 2.8, E 4.5, T 1.9.

Colour in preservative. Base colour of dorsum uniform dark grey, with some small black speckles from base of head to vent. Upper surfaces of limbs same base colour as dorsum and lightly speckled with black. Dark grey base colour of dorsum becomes patchy on lateral aspect of body as base colour is replaced by lighter grey tone, eventually being replaced by flesh colour on venter. Upper lip margin bordered by fine cream line extending from snout to end of jaw where it is expanded. Anterior surface of thigh flesh coloured, without patterning. Venter and undersurfaces of hands and feet cream, throat lightly suffused with light gray wash, edge of mandible dark gray.

Variation. SV of adult ♂♂ (n = 22) measure 38.7–53 mm and ♀♀ (n = 2) 59–63.4 mm. Head length equal to head breadth (HL/HW 1.06±0.06, range 0.94–1.18). Head length approximately ⅓ snout to vent length (HL/SV 0.38±0.02, range 0.35–0.41). Hind limbs short (TL/SV 0.53±0.03, range 0.45–0.57). Eye to naris distance to internarial ratio highly variable (EN/IN 1.16±0.11, range 0.94–1.4). Dorsum lightly shagreened in a majority of specimens. The description of variation of colour in life is based on colour transparencies of three specimens—SAMA R51053, R51055, R51059 (Fig. 3). Dorsum base colour pale brown with dark brown to black speckling variably present. Upper surfaces of limbs have similar colour pattern to dorsum. Loreal region from snout to angle of jaw light green bordered above along canthus rostralis by a black band beginning at snout, passing through eye, across the top of tympanum, over forelimb and broken into black speckling by the intrusion of the dorsal and lateral colours posterior to forelimb. A prominent white stripe along upper lip, starting at snout and continuing for length of jaw, expanding slightly at posterior margin of jaw. Inguinal region, anterior and posterior surfaces of limbs yellow brown or orange. Iris golden.

Etymology. Named in honour of Margaret Davies, Zoology Department, University of Adelaide for her substantial contribution to the advancement of herpetology in Australia and the systematics of Australian anurans.

Distribution and habitat. Known from 14 locations in central-eastern to lower-northeastern New South Wales from north of the Hunter River to the catchment of the Hastings River—a distance of about 150 km (Fig. 1) (NSW NPWS, 1994; Anstis, 1997). All sites were streams above 400 m elevation. Adults are found adjacent to permanently flowing streams, which usually consist of sections of large pools with gentle flow interspersed with faster flowing shallow sections with cascades and waterfalls. On the tablelands the surrounding vegetation may be heath or dry open forest and along the streams the dominant vegetation is tea tree (*Leptospermum* species) with tussocks (*Lomandra* species) and various ferns. Streams on the edge of the escarpment and in deeper gullies are dominated by wet sclerophyll and rain forest vegetation, usually with a rainforest understorey.

Key to the larger members of the *Litoria citropa* species group

1 No supratympanic fold or submandibular gland ..... *Litoria phyllochroa* complex

—— Prominent supratympanic fold and submandibular gland present 2

2 Vocal sac present; adult ♂♂ SV 46.9–56.6 mm, adult ♀♀ SV 56.5–61.8 mm; tympanum obvious; frequent small, raised "warts" with black pigmentation to distal end ..... *L. citropa*

—— Vocal sac absent; hidden tympanum; adult ♂♂ SV 38.7–53 mm, ♀♀ SV 45.8–63.4 mm 3

3 Lightly shagreened skin texture in majority of specimens; colour in life ranges from uniform golden brown with scattered darker mottling over dorsum to specimens with some small areas of green; adult ♂♂ SV 38.7–53 mm, adult ♀♀ SV 59–63.4 mm ..... *L. daviesae*

—— Smooth skin; colour in life predominantly green; adult ♂♂ SV 34.5–40.3 mm, adult ♀♀ SV 45.8–50.4 mm ..... *L. subglandulosa*

Discussion

Litoria daviesae and *L. subglandulosa* represent sibling species that are very similar in external morphology. The species have few mutually exclusive character states. However, the species are clearly genetically well differentiated as exemplified by the divergence at allozyme loci and among mitochondrial nucleotide sequences. The relatively frequent occurrence of similar "cryptic" species pairs or complexes in the eastern Australian anuran fauna (Martin *et al*., 1979; Donnellan *et al*., 1999) suggests the need for a comprehensive screen of wet forest fauna with a combined molecular and morphological approach.

The habitats of *Litoria daviesae* and *L. subglandulosa* are very similar. These species have rarely been detected away from the riparian zone, and the implication is that they rely on habitats within this zone for breeding and foraging. Detailed studies of microhabitat use are necessary to determine the potential affects that changes to riparian habitats may have on the continued persistence of these frogs. Nothing is known about habitat use outside of the breeding season.

Breeding biology. Anstis & Littlejohn (1996) described various aspects of the male advertisement call and oviposition sites of *Litoria daviesae* (specimens from their locations 1–9). When calling, adult males can be found singly or in small numbers at suitable sites along streams. Anstis & Littlejohn (1996) could not distinguish the male advertisement calls of *L. daviesae* (their "southern *L. subglandulosa* localities") and *L. subglandulosa* (their "northern localities"), but they were not explicit in describing how they compared the calls. Aside from small differences in body proportions, the larvae of the two species are apparently similar sharing a larval mouthpart morphology, originally described by Tyler & Anstis (1975), which is unique among Australo-Papuan hylids (Anstis & Littlejohn, 1996).

Assessment of historical and current distribution and conservation status of *Litoria daviesae* n.sp. and *L. subglandulosa*. *Litoria subglandulosa sensu lato* is reported to have declined from sections of its distribution and following assessment of its conservation status (Lunney *et al*., 1996b), was listed as "vulnerable" under the NSW Threatened Species Conservation Act, 1995. In recognizing that this taxon comprises two species, it is desirable to readdress the conservation status of the constituent taxa.

Because most populations of *Litoria daviesae* have been detected only in the past five years (Anstis & Littlejohn, 1996) it is difficult to make a relative assessment of the past and present distribution and abundance of the species. The species is known from 18 different localities. The earliest collections are from near Elands on the Comboyne Plateau and the Williams River in Barrington Tops NP collected in 1972 and 1976 respectively. It is apparent that the species has a restricted distribution. If the species were formerly more widespread it would have been detected more widely as most of the locations from where it is known and areas adjacent to them have had access via forestry roads for many years. It is also apparent that the species is not abundant. Collections from individual locations are small and reports of the species usually refer to small populations (Tyler & Anstis, 1975; Anstis & Littlejohn, 1996). This may, however, be partially due to a restricted breeding period as at six sites in Barrington Tops and Werrikimbe National Park 14–55 frogs were observed in 1993–1996 (Anstis, 1997; present study). Lastly, it appears the species occurs only in a limited range of habitats; there are no records of the adults away from upland streamside habitats, and they do not occur below elevations of about 400 m.

To assess the conservation status of *L. daviesae* n.sp. we used the scoring system reported by Lunney *et al*., (1996a) as follows. The species has a limited distribution. It occurs in a narrow band on the eastern edge of the tablelands and great escarpment of the Great Dividing Range, with a latitudinal range of about 150 km and a narrow east-west distribution. Because previous information on the distribution and abundance of this species is limited, it is difficult to assess whether the species is in decline. However, Anstis (1997) reported reduced population abundance in the region between the Hastings and Manning Rivers. Furthermore, clearing for agriculture has occurred over large areas that were likely to formerly lie within the distribution of this species, particularly in the regions of the Comboyne Plateau and upper Manning River catchment. Forestry occurs at many of the sites where the species is currently known and the short and long-term impacts of various practices have not been assessed. In addition many of the streams within the species' range have been stocked with exotic fish such as carp (*Cyprinus carpio*), trout (*Oncorhynchus* and *Salmo* species) and mosquito fish (*Gambusia holbrooki*), and while there have been no explicit studies of the impact of these fish on the frog there are several studies which indicate they may have a negative impact on eggs and larvae of stream frogs (Harris, 1995; Webb & Joss, 1997). Lastly, this species is a member of a species group that has experienced declines and disappearances from an as yet unidentified cause (Mahony, 1996). The small geographic range of the species, its dependence on unpolluted upland stream habitats (Anstis, 1997), the occurrence of considerable habitat loss and fragmentation within its range, and the apparent isolation of the known populations leads to it being categorized as "vulnerable".

With the recognition of *Litoria daviesae* n.sp., the geographic range of *L. subglandulosa* is reduced. It is now known only from stream habitats on the eastern escarpment of the Great Dividing Range from the "The Flags" near Walcha in the south to Girraween National Park in the north, a distance of about 250 km. Within this range it appears to occur in disjunct populations, usually at high altitude. It is generally associated with well-vegetated, upland streams above 600 m elevation in a similar range of forest types to *L. daviesae* (NSW NPWS, 1994).

We used the scoring system reported by Lunney *et al*., (1996a) in the following assessment of the conservation status of this species. *Litoria subglandulosa* has disappeared from several localities in the south of its range. Despite extensive searches no populations have been detected since 1978 in the New England National Park and surrounding areas (Anstis, 1997), or farther north in the headwaters of the Oban and Henry Rivers. The species was once common there as the species was recorded on numerous occasions in the early 1970s during a period of active field collection (Heatwole *et al*., 1995; Australian Museum Register). Disappearance of frogs from these areas occurred sometime after the mid 1970s. During the past five years the only populations detected in this southern portion of the former distribution were in the eastern catchment of the Guy Fawkes River (present study), in the Warra State Forest east of Ben Lomond, and in the Styx River State Forest (NSW NPWS, 1994). The Northeast Forest Biodiversity Survey conducted by the NSW NPWS from September 1991 to May 1993, which included two spring-summer seasons when the animals are known to be active and breeding, surveyed 573 sites in northeast New South Wales using standardized methods (NSW NPWS, 1994). The region covered by this major survey encompassed the range of *L. subglandulosa sensu lato*, and the habitat of this species was systematically surveyed by nocturnal streamside searches, and included targeted survey methods such as male response to call broadcast. The species was detected on only

eight occasions. In the northern portion of the species range it has been observed in recent years in Gibraltar Range and Washpool National Parks and further north in the Forestlands and Spirabo State Forests and on the Timbarra Plateau (present study; Mahony, unpubl. data). The preferred habitat of the species is the streams that drain east of the Great Dividing Range, in the section of the streams where they begin to descend the great escarpment. The species does not appear to occur any longer in the upper reaches of many of these streams that originate on the tablelands. On the tablelands many streams are predominantly surrounded by cleared agricultural land, are polluted by agricultural and urban by-products, have altered flow regimes and contain introduced predatory fish. The precise impacts of these changes on the frogs and their larval stage are unknown. Combined with its small geographic range, the occurrence of habitat loss and fragmentation within its range, and the apparent isolation of the known populations, leads to *L. subglandulosa sensu lato* being categorized "vulnerable".

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Appendix

Specimens examined. Unmarked specimens were examined for morphology only, specimens marked [a] were examined for morphology and allozyme electrophoresis, specimens marked * were examined for morphology and allozyme electrophoresis and mtDNA, specimens marked [m] were examined for mtDNA only. Abbreviations in bold refer to OTU codes mentioned in the text. Institutional abbreviations follow Leviton *et al*. (1985) except for ABTC—Australian Biological Tissue Collection, South Australian Museum, Adelaide. All locations are in New South Wales unless indicated otherwise.

Litoria citropa: Endrick R. SAMA R42608*, R42609*; Wombeyan R. ABTC 7146[m] (no voucher).

***Litoria pearsoniana*-1**: Emu Ck, Qld SAMA R33652[m].

***Litoria pearsoniana*-2**: Doyles River SF ABTC 26055* (no voucher); Allyn R. ABTC 25688[m].

Litoria phyllochroa: Roseville ABTC 25677[m].

Litoria nudidigitus: Aberfeldy R., Vic. ABTC 60187[m].

Litoria subglandulosa: **Girr**: Girraween, Qld QM J31487–9, ABTC 68396[m], 68397[m], 68398[m] (no vouchers); **Timb**: Timbarra Plateau SAMA R51051*; **Gibr**: Dandahra Creek, Gibraltar Range NP SAMA R39108*; Gibraltar Range NP QM J31490–2, J40012, J55262–3; **Dorr**: Wild Cattle Creek SAMA R51052*; Sandys Ck Dorrigo AMS R52391; Barwick Creek, near Ebor SAMA R13303, R13504–10, R13060, QM J17025, J27495; 11km S Ebor QM J18044; Styx River QM J26028; Little Styx River, near Point Lookout SAMA R13626–39, R13677–80; Point lookout AMS R17577, R42933, R51097, R51104, R51736–7, R51739–41, R52630; The Flags AMS R37017.

***Litoria daviesae* n.sp.**: **Werr**: Werrikimbe National Park SAMA R51053*, R51054*, AMS R153052*; Mt Boss SF AMS R108692; **Doyle**: Doyles River SAMA R51055, ABTC 26058*; **Royal**: Mount Royal SAMA R51056*, R51057*, R51058*; **Ellen**: Ellenborough River SAMA R51059*; Bulga SF AMS R104932; **Elands**: Elands ABTC 7088[a] (no voucher); Sharpes Creek, Gloucester Tops SAMA R51060* (typed for diagnostic loci only); Barrington Tops SF AMS R76519–20; **Dilg**: Dilgry R. SAMA R51061*, R510612*, R510613*, AMS R148831; Upper Allyn River AMS R31683; Williams River, AMS R144861–2; Dingo Tops SF AMS R148855; Tuggalo SF AMS R150090. **Specimens not assigned**: New England NP AMS R35525, Oaky Ck AMS R36724, Gibraltar Range NP AMS R96910, Forbes R. AMS R103080.

Litoria spenceri: Big River, Vic. SAMA R47504*; White Creek, Vic. SAMA R45359*; Howqua River, Vic. SAMA R47485*; Bundarra R., Vic. SAMA R43767[m], Bogong Creek SAMA R47674*.

Litoria caerulea: SAMA R33448 Townsville, Qld.