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ORIGINAL PAPER

Conservation genetics and species status of an endangered Australian dragon, *Tympanocryptis pinguicolla* (Reptilia: Agamidae)

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Abstract We present a phylogenetic and morphological study of the grassland earless dragon, *Tympanocryptis pinguicolla*, an endangered habitat specialist that occurs in a few isolated populations in eastern Australia. *Tympanocryptis pinguicolla* occurred historically in Victoria in south-eastern Australia, but has not been seen since 1990, and current populations are known in New South Wales and Canberra in south-eastern Australia. Recently, new populations identified as *T. pinguicolla* were discovered on the Darling Downs, Queensland. Translocation of individuals between these populations has been suggested as a conservation management strategy to maintain genetic diversity. To address this issue, we undertook a phylogenetic study of all major populations of *T. pinguicolla* using a 1838 bp region of mitochondrial DNA, incorporating *ND1*, *ND2*, *COI* and eight tRNA genes. We incorporated specialized degraded-DNA techniques to amplify DNA from historical museum specimens, as no extant tissue was available for Victorian populations. Our results, which include morphological analysis, provide convincing evidence that populations currently identified as *T. pinguicolla* do not comprise a monophyletic species, as the populations from the Darling Downs are more closely related to *T. tetraporophora* than to *T. pinguicolla*. In addition, we find that there is a significant level of haplotype divergence between populations of *T. pinguicolla*, indicating that these lineages separated at least 1.5 mya. Our results suggest translocation may not be an appropriate management strategy and our findings that Darling Downs populations are not *T. pinguicolla* will significantly influence the conservation management of this species in Queensland.

Keywords Agamidae · Genetic diversity · Haplotype divergence · Historical biogeography · Reptiles

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Introduction

Prior to European settlement in the late 1700's, there were extensive areas of native grasslands in south-eastern Australia (Fig. 1) but in the last two centuries they have diminished significantly due to agriculture and urbanization (Driscoll and Hardy 2005). Areas of remnant native grasslands are found around eastern Australia's largest cities but are highly modified and fragmented. For example, west of Melbourne the Western Plains Grassland is recognized as one of the most endangered vegetation communities in the state of Victoria (Stuwe 1986; Frood and Calder 1987).

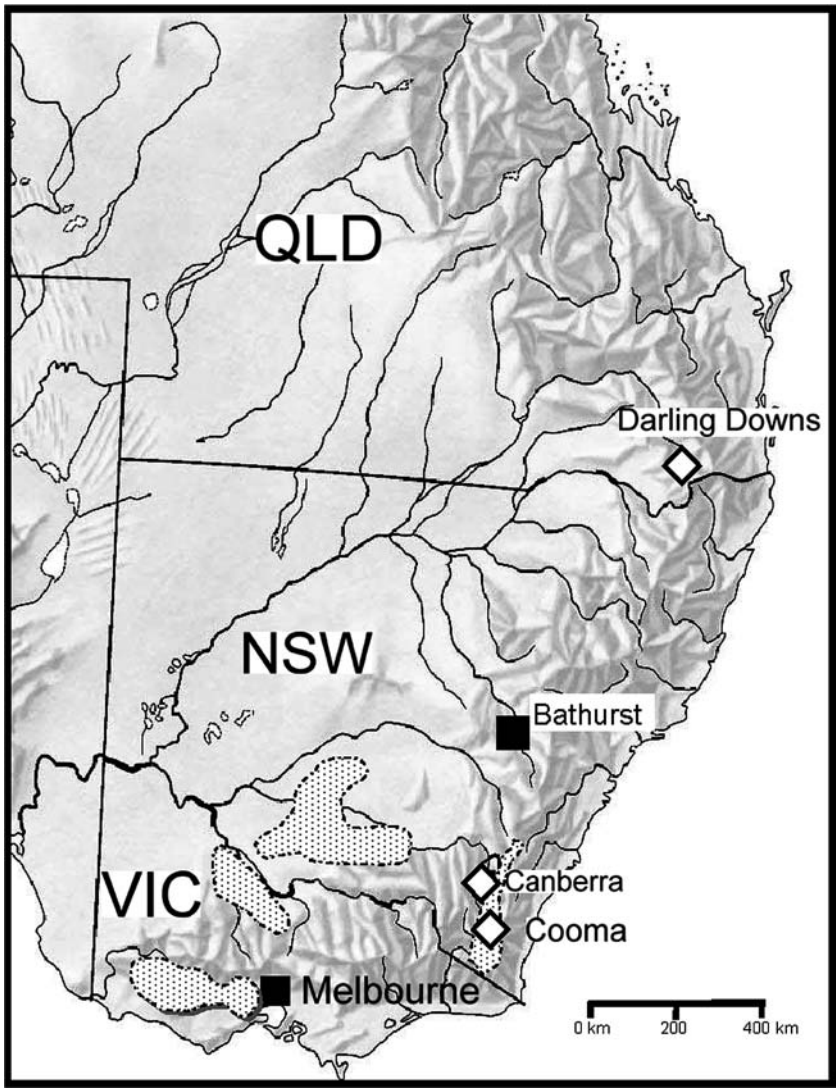

Fig. 1 Map of Australia's eastern states: Queensland (QLD); New South Wales (NSW); and Victoria (Vic). Estimated distribution of temperate lowland grasslands prior to European settlement (circa. 1770) are indicated by stippled areas enclosed by dotted lines (Lunt 1995). Distribution of current (◇) and historical (■) populations of *Tympanocryptis pinguicolla* are provided (Robertson and Cooper 2000)

Many of the vertebrate species that occur in Australia's temperate grasslands, such as legless lizards, small marsupials and grassland birds, are habitat specialists and restricted to grasslands (Stuwe 1986). This specialization means that as grasslands become increasingly fragmented, many populations will become isolated, diminish in size and be at increased risk of localized extinction.

Translocation of individuals between fragmented populations in species that once had more continuous distributions is a commonly cited method to counteract the deleterious effects of habitat and population fragmentation (Frankham et al. 2002). However, without prior knowledge of the genetic structure of species, there is a risk of drastically altering the genetic structure of populations and potentially altering the evolutionary course of a species (Moritz 2002). Thus, detailed knowledge of the phylogeography and population genetic structure of a species is essential prior to the initiation of any translocation conservation program. We investigate these concepts with a small earless dragon, *Tympanocryptis pinguicolla*, from the temperate grasslands of south-eastern Australia.

Tympanocryptis pinguicolla is a habitat specialist, occurring only in grasslands and using spider burrows as retreat sites (Smith et al. 1999; Scott and Keogh 2000). The dramatic reduction in native grasslands has meant that suitable habitat for *T. pinguicolla* has greatly diminished and the species is now formally listed as endangered throughout its range (Robertson and Cooper 2000). In Victoria, five sightings believed to be this species were reported between 1988 and 1990 but none since, while approximately 500 kms north in New South Wales (NSW) and Canberra the species survives in only a few locations (Fig. 1). In 2001 an individual identified as *T. pinguicolla* was captured on the Darling

Downs, Queensland (QLD), which is more than 1000 km north of the populations in Canberra and NSW. Since then a number of new populations have been located in the Darling Downs (QLD) region (Hobson 2002; Starr and Leung 2005). Due to the highly fragmented nature of these remaining populations, which precludes gene flow among them, it has been suggested that translocation of individuals may be a viable option to avoid inbreeding and loss of genetic diversity (Robertson and Cooper 2000). However, a preliminary molecular study (Scott and Keogh 2000) of the NSW and Canberra populations suggested a significant level of genetic divergence (>5%) over relatively small geographic scales, which pre-dates European settlement and subsequent habitat reductions.

We undertook a phylogenetic study of all known populations of *Tympanocryptis pinguicolla* to determine the extent of genetic divergence, with particular emphasis on the recently discovered populations from the Darling Downs (QLD) and extirpated populations in Victoria. An additional seven species of *Tympanocryptis* and two outgroup taxa are included in the analysis. Sequences reported include a 1838 base-pair (bp) segment of the mitochondrial genome spanning the ND1, ND2 and COI genes and intervening tRNA genes. As no extant tissue samples were available for Victorian populations of *T. pinguicolla*, we use specialized degraded-DNA techniques on spirit-preserved museum specimens to provide a 331 bp section of the ND2 protein-coding gene for analysis. We also incorporated a morphological analysis of populations from the Darling Downs in Queensland and Canberra/NSW to determine whether phylogenetic divergences are supported by distinguishable morphological characters. In addition, we test hypotheses concerning the phylogenetic relationships and species status of *T. pinguicolla* populations.

Materials and methods

Genetic and morphological samples

A total of 18 genetic samples of *Tympanocryptis pinguicolla* were included in this study from four regions (Appendix 1), including: eight from Cooma (NSW); three from Canberra; six from Darling Downs (QLD); and one from Melbourne (Victoria). No extant tissues were available from Victoria, as the last individuals were sighted in 1990 and no tissues were collected. Thus we used specimens held in the Museum Victoria collections that pre-dated the use of formalin. Of three specimens sampled from Museum Victoria, one yielded viable DNA using specialized techniques described below. All other samples of *T. pinguicolla* used in this study were tail tips or toe clips collected during field surveys. Our analyses also included 16 previously published sequences (Appendix 1: Melville et al. 2001; Schulte et al. 2003).

Animals used for the morphological analysis were processed at the Queensland Museum. A total of 18 specimens of *T. pinguicolla* from museum collections were used: seven from the Darling Downs, QLD and eleven from Canberra/NSW (Appendix 1). In addition, 13 live specimens were collected from private property from known populations of *T. pinguicolla* at Bongeen on the Darling Downs, QLD.

Morphological measurements and data analysis

Eighteen morphological measurements and meristic counts were collected for all specimens using 0–200 mm Digital Vernier calipers (Scientific Instrument and Optical sales). Morphological measurements included: snout-vent length (SVL), tail length (TL), head length (HL), head width (HW), head depth (HD), axilla – groin length (AG), fore limb length (FLL), hind limb length (HLL), width of enlarged dorsal spinous scales (SSW), length of enlarged dorsal spinous scales (SSL), number of inter-nasals (IN), number of infralabials (IL), number of supralabials (SL), number of scales between nasal and supralabial (NL), number of spinous scales between axilla and groin (AXG), number of enlarged dorsal spinous scales (DSS), number of preanal pores (PP), and number of femoral pores (FP).

The differences between populations were analyzed using an analysis of variance (ANOVA). Due to the unbalanced nature of the data (due to unequal numbers of animals in each population/location) a general linear regression approach was used. Pair-wise comparisons were used to identify significant terms using a *t*-test. The relationship between morphological measurements were analyzed using the following: length of enlarged dorsal spinous scales to width of enlarged dorsal spinous scales (SSL/SSW), hind limb length to snout-vent length (HLL/SVL), head length to head depth (HL/HD), and number of spinous scales between axilla and groin to number of enlarged dorsal spinous scales (AXG/DSS). The raw data of all meristic characters were analyzed. Residual plots were used to ensure normality of the data and measurements did not need transforming. The number of preanal pores (PP) and the number of femoral pores (FP)

measurements were excluded from analysis as they contained little or no variation.

All morphological and meristic data were also analyzed using principal components analysis (PCA), except for preanal pores (PP) and femoral pores (FP) as the data contained little variation. The principal components were extracted from the correlation matrix of the raw data of all species. Normality of all variables was ensured prior to analysis. Three principal components (henceforth PCs) were utilized in the analysis, which was determined using the elbow criterion of eigenvalues plotted against factors, maximizing the adequacy of extraction.

Laboratory protocols and alignment of DNA sequences

Genomic DNA was extracted from tail tips or toe clips using a modified CTAB procedure (Scott and Keogh 2000) or a salt precipitation procedure (Nicholls et al. 2000). Two overlapping fragments (~1100–1500 bp) of the mtDNA genome extending from the gene encoding ND2 (NADH dehydrogenase subunit two), through the genes encoding $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, O_L (origin of light-strand synthesis), $tRNA^{Cys}$, $tRNA^{Tyr}$, to the protein-coding gene, *COI* (subunit I of cytochrome c oxidase) were amplified using primers L3914 (incorrectly labeled L3878 in Macey et al. 1998)/H4980 (Macey et al. 1997a) and L4437b (Macey et al. 1997a)/H5934 (Macey et al. 1997a). Amplifications were performed in 25 μl volumes in the presence of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of forward and reverse primer, 1× Qiagen PCR buffer and 1 Unit of HotStarTaq DNA polymerase (Qiagen). Thermal cycling conditions consisted of an initial denaturing and enzyme activation step at 95°C for 15 min followed by 35 cycles of denaturing at 95°C for 20 s, annealing at 50°C (or 55°C) for 20 s, and extension at 72°C for 90 s, using a Corbett thermocycler.

Genomic DNA was extracted from small pieces of muscle tissue harvested from ethanol preserve museum specimens using a DNeasy tissue extraction kit (Qiagen) as per manufacturer's instructions. All pre-PCR work was done in a physically separate laboratory dedicated to ancient DNA work to eliminate contamination (see Austin et al. 1997a, b for contamination and authentication controls). Three short (113–125 bp, excluding primers) fragments of the ND2 gene were amplified using primers L4437b (Macey et al. 1997a)/ND2R.696 (5′-TTTAATTCYAGGCCKAGTCAGGC-3′, this study), ND2F.679 (5′-GCACACTAACAGTTACCTCAGC-3′, this study)/ND2R.804 (5′-ATGAGTGCGGAGGCAGTTGC-3′, this study) and ND2F.1012 (5′-CAGCCCTAYTAATYACAACCTG-3′, this study)/H4980 (Macey et al. 1997a). Amplifications were performed using identical PCR conditions as for extant tissues, however after the initial PCR amplification, 1 μl of product was used to spike a second PCR reaction using the same primers and PCR conditions.

All successful amplifications were purified by isopropanol precipitation and sequenced in both directions by a commercial company (Macrogen, Korea) using the same forward and reverse primers used in the PCR. Sequence chromatograms were edited and a single contiguous sequence for each specimen produced using Sequencher (Gene Codes Corporation, USA). DNA sequences were aligned using tRNA secondary structure models (Macey et al. 1997b) and amino acid translations (for protein coding regions).

Phylogenetic analyses

Sequence from Victorian *T. pinguicolla* (D3483) consisted of a 331 bp section of the ND2 protein coding gene amplified from an historical museum specimen. Inclusion of this specimen in analyses may have influence on branch support and hypothesis testing due to large amounts of missing data. Thus, we conducted all analyses twice, including and excluding sample D3483. PAUP* beta version 4.0b410a (Swofford 2002) was used to estimate phylogenetic trees with 100 heuristic searches using random addition of sequences under the parsimony criterion. Bootstrap resampling was applied to assess support for individual nodes using 1000 bootstrap replicates with 10 heuristic searches featuring random addition of sequences. ModelTest version 3.06 (Posada and Crandall 1998) was used to compare goodness-of-fit of different models of sequence evolution to the data, and to generate optimal settings for a maximum-likelihood estimate of phylogeny, using the standard AIC criterion. The best-fitting model parameters were fixed and the overall most parsimonious tree(s) used as starting trees for branch swapping in 25 heuristic searches with random addition of taxa to find the overall best likelihood topology. Bootstrap resampling was applied to assess support for individual nodes using 100 bootstrap replicates with 10 heuristic searches featuring random addition of sequences. Model parameters were fixed for bootstrap analyses, as above.

Bayesian analysis was used to estimate a phylogenetic tree using many of the default values in MrBayes 2.1 (Huelsenbeck and Ronquist 2001). All analyses were initiated from random starting trees and run for

2,000,000 generations using four incrementally heated Markov chains. Values of the likelihood model selected from the best-fit model of nucleotide substitution using ModelTest were estimated from the data and initiated using flat priors. Trees were sampled every 100 generations resulting in 20,000 saved trees. To ensure that Bayesian analyses reach stationarity, the first 5,000 saved trees were discarded as ''burn-in'' samples following Leaché and Reeder (2002). Three analyses were run independently beginning with different starting trees to check that searches did not become trapped on local optima. For all three runs ln-likelihood scores converged on similar values. Sampled trees from all three runs were combined to yield 45,000 saved trees. These trees were used to generate a 50% majority-rule consensus tree in PAUP*, and the percentage of trees having a particular clade represented that clade's posterior probability (Huelsenbeck and Ronquist 2001).

The SH test (Shimodaira and Hasegawa 1999), a likelihood-based test of topologies, was used to evaluate the statistical significance of the favored tree relative to alternative hypotheses. The statistical power of the SH test appears comparable to that of the two-tailed Wilcoxon signed-ranks test (Townsend and Larson 2002). Phylogenetic topologies were constructed in MacClade (Maddison and Maddison 1992) and used as constraints in PAUP*. A search using the maximum-likelihood settings determined previously in MODELTEST and starting from a neighbor-joining tree was used to estimate a phylogenetic tree that maximized the likelihood of the alternative hypothesis. An SH-test, using 10,000 replications, was then run to determine whether the favored likelihood tree is significantly better than an alternative or whether their differences are attributed to chance alone.

Results

Sequence alignment

Nineteen new sequences representing ~1838 bp of the mitochondrial genome are reported for two species of *Tympanocryptis*: *T. tetraporophora* (one sample) and *T. pinguicolla* (18 samples). These sequences are aligned for phylogenetic analysis with previously published sequences: seven *Tympanocryptis* species and two outgroup sequences from *Pogona vitticeps* and *Rankinia diemensis*. Of the 1616 unambiguous sites in 27 aligned sequences, 531 are variable and 349 are parsimony informative.

All base positions in protein-coding genes are alignable. The following loop regions among tRNA genes and non-coding regions between genes are unalignable and were excluded from analyses: parts of the dihydrouridine (D) and the TYC (T) loops for the genes encoding $tRNA^{Ile}$ (positions 201–208, 240–249), $tRNA^{Trp}$ (positions 1385–1393, 1408–1418) and $tRNA^{Tyr}$ (positions 1721–1728, 1741–1747), parts of the D-loop from the genes encoding $tRNA^{Asn}$ (positions 1573–1578), $tRNA^{Ile}$ (positions 202–208) and $tRNA^{Gln}$ (positions 154–161), the T-loops from the genes encoding $tRNA^{Met}$ (positions 324–326) and $tRNA^{Cys}$ (positions 1665–1672), parts of the variable loops from the genes encoding the $tRNA^{Asn}$ and $tRNA^{Cys}$ (positions 1544–1548, 1665–1672), the D-stem and associated loop region of the $tRNA^{Cys}$ gene (positions 1678–1682), non-coding sequences between the ND1 and $tRNA^{Gln}$ genes (positions 76–100), the $tRNA^{Gln}$ and $tRNA^{Ile}$ genes (positions 175–187), ND2 and $tRNA^{Met}$ genes (positions 340–346), the $tRNA^{Trp}$ and $tRNA^{Ala}$ genes (positions 1450–1457), the $tRNA^{Asn}$ and $tRNA^{Cys}$ genes (positions 1602–1609), and the $tRNA^{Cys}$ and $tRNA^{Tyr}$ genes (positions 1700–1713). Excluded regions comprise 222 of the 1838 aligned positions.

Phylogenetic inferences using parsimony, maximum-likelihood and Bayesian analyses

Analyses that incorporated or excluded sample D3483 had identical topology and very similar branch support (Fig. 2a, b). In addition, maximum parsimony, maximum-likelihood and Bayesian analyses converged on congruent topologies with the same branches receiving bootstrap and Bayesian posterior probability support. We use Bayesian and likelihood-based methods for further analyses.

Analyses that excluded Victorian *T. pinguicolla* (D3483) found that likelihood model TVM+G has the highest likelihood and is significantly favored over 56 alternative likelihood models. Model parameters are: $\alpha = 0.243$; proportion of invariant sites = 0; substitution rates R(a) = 1.229, R(b) = 13.114, R(c) = 0.705, R(d) = 0.680, and R(e) = 13.114; and empirical base frequencies A = 0.340, C = 0.313, G = 0.114, and T = 0.233. A single optimal tree with a ln-likelihood of –6510.20 was found. (Fig. 2a). Bayesian analysis performed using the TVM+G nucleotide-substitution model and parameters estimated from the sequence data produced a consensus tree with the same topology as the maximum-likelihood tree (mean ln-likelihood = –6589.54 and variance of 48.73, Fig. 2a).

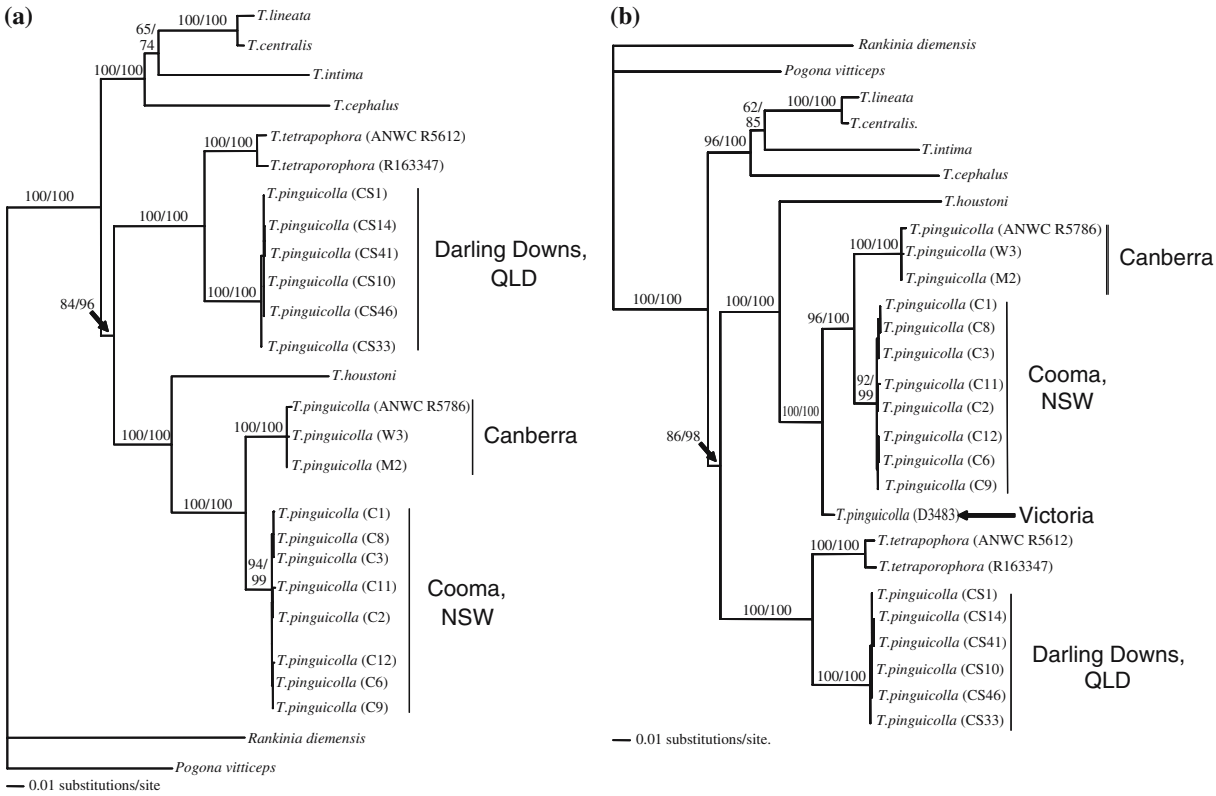

Fig. 2 Optimal maximum-likelihood tree under the TVM+G model, which indicates phylogenetic relationships within *Tympanocryptis pinguicolla* (**a**) excluding the historical sample from Museum Victoria collection (D3483) and (**b**) including the historical sample from Museum Victoria collection (D3483). Values presented above the branches are as follows: maximum likelihood bootstrap values are presented first and Bayesian posterior probability values are presented second

Likelihood analyses that included Victorian *T. pinguicolla* (D3483) determined that the model TVM+G has the highest likelihood. Model parameters are: $\alpha = 0.243$; proportion of invariant sites = 0; substitution rates R(a) = 1.1369, R(b) = 12.315, R(c) = 0.668, R(d) = 0.598, and R(e) = 12.315; and empirical base frequencies A = 0.340, C = 0.314, G = 0.115, and T = 0.231. A single optimal tree with a ln-likelihood of –6543.31 was found (Fig. 2b). Bayesian analysis performed using the TVM+G nucleotide-substitution model and parameters estimated from the sequence data produced a tree with the same topology as the maximum-likelihood tree, with a mean ln-likelihood of –6673.20 and variance of 51.23 (Fig. 2b).

Maximum-likelihood bootstrap resampling using 100 bootstrap replicates and Bayesian posterior probabilities (Fig. 2a, b) does not support the monophyly of sampled populations of *Tympanocryptis pinguicolla*. Rather *T. pinguicolla* from Darling Downs (QLD) are strongly supported as being most closely related to *T. tetraporophora*, while Victorian, Canberra and Cooma (NSW) populations are strongly supported as being most closely related to *T. houstoni*. The clade containing *T. pinguicolla* from the Darling Downs (QLD) and *T. tetraporophora* receives very strong branch support, as does the clade containing Victorian, Canberra and Cooma (NSW) populations of *T. pinguicolla* and *T. houstoni* (Fig. 2a, b). Statistical SH-tests strongly reject the monophyly of *T. pinguicolla* (Table 1).

The Darling Downs (QLD) populations of *Tympanocryptis pinguicolla* form a strongly supported monophyletic clade (Fig. 2a, b), although little resolution is provided within this region. The Cooma (NSW) population of *T. pinguicolla* is also well supported as a monophyletic clade, as are the Canberra populations. Statistical tests significantly support the monophyly of these populations (Table 1). Topology (Fig. 2b) supports that the Victorian population is basal in *T. pinguicolla* (incorporating Victoria, Cooma (NSW) and Canberra), with the Cooma (NSW) and Canberra populations being sister clades. This topology receives strong branch support, however, as this relationship is based on a

Table 1 Phylogenetic hypotheses tested using the SH test with molecular data

Hypothesis	SH-test	
	Diff – ln L	*P*
A. *Monophyly of*		
1. *T. pinguicolla* (including Vic. D3483)	90.78	< 0.001
2. *T. pinguicolla* (excluding Vic. D3483)	85.59	< 0.001
B. *Non-monophyly of*		
1. Canberra pop. of *T. pinguicolla* (including Vic. D3483)	23.64	0.001
2. Canberra pop. of *T. pinguicolla* (excluding Vic. D3483)	20.25	0.004
3. NSW pop. of *T. pinguicolla* (including Vic. D3483)	12.06	0.043
4. NSW pop. of *T. pinguicolla* (excluding Vic. D3483)	12.04	0.047

The null hypothesis is that the maximum-likelihood tree corresponding to conditions listed below is not significantly different from the overall optimal maximum-likelihood tree. (Diff – ln L is the difference in ln-likelihood between the alternative hypothesis as stated and the overall maximum-likelihood tree)

single specimen from Victoria, further sampling is required to confirm this relationship.

Morphological differences between populations

A general linear regression of the proportional morphological measurements and meristic counts (Table 2) show that there are significant differences between the Darling Downs (QLD) and Canberra/NSW populations of *Tympanocryptis pinguicolla* (Table 3). The results demonstrate that specimens of *T. pinguicolla* from the Darling Downs (QLD) have larger individual spinous scales (only being slightly longer than wide), longer and shallower dimensions in

Table 2 Morphological measurements (a) and meristic characters (b) that showed variation between the Darling Downs (QLD) and Canberra/NSW populations of *Tympanocryptis pinguicolla*

Attribute	Location	Minimum (mm)	Maximum (mm)	Mean (mm)	StdDev	*N*
(a) Morphological measurements						
SSL	Qld	0.74	1.29	1.05	0.18	20
	Canberra	0.68	1.1	0.92	0.15	11
SSW	Qld	0.71	1.28	1.02	0.17	20
	Canberra	0.48	0.69	0.59	0.07	11
HL	Qld	11.39	16.97	13.97	1070	20
	Canberra	10.84	16.30	13.34	1072	11
HD	Qld	6.59	10.11	8.10	0.99	20
	Canberra	6.95	10.41	8.57	1.13	11
HLL	Qld	25.71	47.78	35.39	5.89	20
	Canberra	27.46	40.31	34.25	3.99	11
Attribute	Location	Minimum (counts)	Maximum (counts)	Mean (counts)	StdDev	*N*
(b) Meristic characters						
AXG	Qld	7	16	10.60	1.98	20
	Canberra	0	0	0.00	0.00	11
DSS	Qld	32	42	35.85	3.27	20
	Canberra	44	69	57.73	8.46	11
IL	Qld	9	11	10.30	0.66	20
	Canberra	10	14	11.91	1.30	11
SL	Qld	9	11	10.30	0.66	20
	Canberra	10	13	11.18	1.08	11
PP	Qld	2	2	2.00	0.00	20
	Canberra	2	2	2.00	0.00	11
FP	Qld	0	2	0.20	0.62	20
	Canberra	0	0	0.00	0.00	11

Abbreviations: tail length (TL), head length (HL), head depth (HD), hind limb length (HLL) width of enlarged dorsal spinous scales (SSW), length of enlarged dorsal spinous scales (SSL), number of infralabials (IL), number of supralabials (SL), number of spinous scales between axilla and groin (AXG), number of enlarged dorsal spinous scales (DSS), number of preanal pores (PP), and number of femoral pores (FP)

Table 3 Results of general linear regression analysis of proportional morphological measurements and meristic characters between the Darling Downs (QLD) and Canberra/NSW populations of *T. pinguicolla*

Measure	Predicted means		*t*-probability
	Queensland	Canberra	
IL	10.30	11.97	***
SL	10.31	11.20	***
AXG	10.61	0	***
DSS	1.02	0.58	***
HL/HD	1.70	1.56	***
HLL/SVL	0.74	0.69	***
SSL/SSW	1.03	1.59	***
AXG/DSS	0.299	0.00	***

Significance was determined using pair-wise comparisons *** = $P < 0.001$)

Abbreviations: snout-vent length (SVL), head length (HL), head depth (HD), hind limb length (HLL), width of enlarged dorsal spinous scales (SSW) and length of enlarged dorsal spinous scales (SSL), number of infralabials (IL), number of supralabials (SL), number of spinous scales between axilla and groin (AXG), number of enlarged dorsal spinous scales (DSS)

Table 4 Percent variance and component loadings for principal components analysis of morphological measurements and meristic character counts

Percent variance	Factor 1 Loadings	Factor 2	Factor 2
	49.26	12.27	9.98
SVL	–0.34	–0.07	0.06
TL	–0.32	–0.02	–0.01
HL	–0.32	–0.05	0.10
HW	–0.34	–0.05	0.04
HD	–0.31	–0.14	0.01
HLL	–0.31	0.01	0.20
FLL	–0.30	–0.01	0.03
AG	–0.31	0.01	0.02
SSL	–0.23	0.10	0.29
SSW	–0.27	0.33	–0.02
IN	0.05	–0.34	0.16
IL	–0.14	–0.19	–0.59
SL	–0.12	–0.20	–0.58
NL	–0.13	–0.25	–0.22
AXG	–0.06	0.53	–0.18
DSS	0.04	–0.55	0.27

Abbreviations: snout-vent length (SVL), tail length (TL), head length (HL), head width (HW), head depth (HD), axilla–groin length (AG), fore limb length (FLL), hind limb length (HLL) width of enlarged dorsal spinous scales (SSW), length of enlarged dorsal spinous scales (SSL).Number of inter-nasals (IN), number of infralabials (IL), number of supralabials (SL), number of scales between nasal and supralabial (NL), number of spinous scales between axilla and groin (AXG), and number of enlarged dorsal spinous scales (DSS)

head tape and a greater mean hind leg length than specimens from Canberra/NSW. Meristic character counts indicated that true spines were present on the axilla groin line of Darling Downs (QLD) specimens as opposed to Canberra/NSW specimens, which lacked any significant spines along this line. The dorso spinous scale count in *T. pinguicolla* from the Darling Downs (QLD) was significantly less than Canberra/NSW populations, while specimens from the Darling Downs (QLD) have an overall lower count of supralabials and infralabials. All morphological and meristic data were included in analyses, except for the presence/absence of preanal pores and femoral pores as there was little variation across all individuals examined (Table 2).

A principal components analysis of the morphological and meristic data, which is reported in Table 2, accounts for 71.5% of the variation in the raw data (Table 4). The loadings for PC1 were comprised predominantly of body size and shape measurements, which were not found to vary significantly between populations of *T. pinguicolla*. However, PC2 scores of individuals from the Darling Downs (QLD) and Canberra/NSW populations were found to differ significantly ($F_{1,29} = 166.10$, $P < 0.001$). The second principal component showed strong positive loading of spinous scale width (SSW), internasal scale count (IN), axilla groin scale count (AXG) and dorso spinous scale count (DSS). The third principal component, which loaded strongly for scale length (SSL), infralabial scale count (IL) and supralabial scale count (SL), did not vary significantly between the Darling Downs (QLD) and Canberra/NSW populations of *T. pinguicolla*.

Discussion

Phylogenetic relationships, species status and biogeography

Our results do not support the monophyly of populations of grassland earless dragons currently assigned to *Tympanocryptis pinguicolla*. We provide strong evidence based on both genetic and morphological data that the recently discovered populations of putative *T. pinguicolla* from the Darling Downs region of Queensland are not the same species. Populations from the Darling Downs (QLD) and the Canberra/NSW could be distinguished morphologically for a number of characters, including scalation and body proportions. Our genetic data shows that putative *T. pinguicolla* from Queensland are more closely related to *T. tetraporophora* than they are to *T. pinguicolla*. Haplotype divergences between the Darling Downs (QLD) animals and populations of *T. pinguicolla* are

significant (11.3–12.2% uncorrected genetic distance). Using a molecular clock of 1.3% divergence between lineages per million years (Macey et al. 1999; Weisrock et al. 2001) or even a more conservative estimate of 2% per million years (Brown et al. 1979; Wilson et al. 1985), we estimate that these two clades diverged more than 5.5 mya. Our study is not able to determine whether animals from the Darling Downs (QLD) represent a sister taxa to *T. tetraporophora* or is synonymous with this species but wider geographic sampling of *T. tetraporophora* should resolve this issue. However, our study does suggest that a taxonomic revision of the putative *T. pinguicolla* from the Darling Downs (QLD) is required. We propose that until further taxonomic work is completed that it be referred to as *T. c.f. tetraporophora*.

Our results for *Tympanocryptis pinguicolla sensu strictu* (i.e. Cooma, Canberra and Victorian populations) are consistent with preliminary molecular phylogenetic work by Scott and Keogh (2000), which examined the genetic divergence between Cooma (NSW) and Canberra populations. This previous study, which used the ND4 mitochondrial region, found significant levels of genetic divergence between the two populations (>4.8%—Jukes-Cantor corrected distance). Similarly, we found 3.4–3.7% uncorrected genetic difference between these populations. Both these studies provide strong evidence that the divergence between these populations pre-date European settlement, indicating that these populations have existed as geographically isolated populations for significant amount of time. Using a molecular clock estimate of 1.3% divergence between lineages per million years (Macey et al. 1999; Weisrock et al. 2001) or a more conservative estimate of 2% per million years (Brown et al. 1979; Wilson et al. 1985), these two populations diverged more than 1.8 mya. The genetic distance between the Victorian sample included in this study and the Cooma and Canberra samples are even greater (3.9–5.2%). Although we were only able to extract DNA from one specimen, our study provides initial evidence that these populations have been genetically isolated for at least 2 million years.

The substantial genetic divergence (3.4–12.2%) between and within the *Tympanocryptis tetraporophora* clade and the *T. houstoni/pinguicolla* clade suggests that these groups have a long evolutionary history dating to the Pliocene and late Miocene, between 2 and 8 mya. This period coincides with major climatic and vegetational changes in south-eastern Australia. In response to increasing coolness and dryness, forests of the Miocene gave way to extensive woodlands, grasslands and deserts. Palynological records have shown that grasses first appeared in Australia during the Oligocene but were restricted to the north-western portion of the continent (Kershaw et al. 1994). The spread of open vegetation and grasslands into central and south-eastern Australia accelerated in the late Miocene, with increased seasonality and a cooler climate. Although pollen records are poor during the Pliocene it is believed that open woodlands and grasslands continued to expand (Kershaw et al. 1994).

During the late Pliocene (approximately 2.5 mya) further changes in the south-east Australian climate saw the development of winter rainfall systems and the development of glacial–interglacial sequences where the climate oscillated between short, warm periods and longer cool periods (Bowler 1982). It is believed that expansion and contractions of woodlands and grasslands occurred during the glacial oscillations of the late Pliocene and the Pleistocene (Kershaw et al. 1994). It is probable that these climatic and vegetational changes shaped the inter-populational haplotype divergences in *Tympanocryptis pinguicolla sensu strictu*, where populations became isolated with little or no gene flow early on in their evolutionary history. This level of haplotype divergence has been compared to species-level taxonomic status in other species (Scott and Keogh 2000) and has significant implications for conservation management.

Conservation implications

The convincing genetic evidence in our study that the Darling Downs (QLD) populations of *Tympanocryptis pinguicolla* are in fact either a sister species to or synonymous with *T. tetraporophora* has major conservation management consequences. This taxonomic distinction is important because *T. pinguicolla* is federally listed as endangered, while *T. tetraporophora* is not listed as a species of concern in Queensland. A considerable level of research and conservation effort has been focused on these Queensland populations of *T. pinguicolla* by government agencies, community groups and non-profit conservation organizations. Thus, our study will significantly impact future conservation policy for putative *T. pinguicolla* in Queensland, as we found no evidence that this species occurs in the state. However, these results do not preclude *T. pinguicolla* being located in future surveys of suitable grassland habitats in Queensland. In addition, although our study shows that these populations are not closely related to *T. pinguicolla*, we are not able to establish whether they are an undescribed species of *Tympanocryptis* or a population of

T. tetraporophora. If ongoing molecular and taxonomic research demonstrates that these populations are an undescribed species, a re-assessment of its conservation status would be required.

Within *Tympanocryptis pinguicolla sensu strictu* (i.e. Cooma (NSW), Canberra and Victorian populations) the significant level of haplotype divergence between the three population groups provides strong evidence of a long history of genetic isolation. These genetic divergences pre-date recent human induced changes in the vegetation. It is probable that decreases in suitable habitat through agriculture and urbanization has changed the local genetic structure within this species but our study shows that the major genetic structure between Cooma (NSW), Canberra and Victorian populations date back to the late Pliocene. For this reason translocation of individuals between these populations could detrimentally impact this species in a number of ways (Moritz 2002). For example, inter-breeding between translocated individuals would dramatically and artificially alter the genetic structure of this species and hybridization between the genetically divergent haplotypes may result in lowered fitness of offspring. Consequently, the results of our study should be taken into consideration for conservation management strategies involving the translocation of individuals between the major populations (Cooma (NSW), Canberra and Victorian) of *T. pinguicolla*.

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Appendix 1

Samples for DNA sequencing

Museum or field collection numbers and localities of specimens from which DNA was extracted are provided below (NOTE: GenBank accession numbers will be provided after paper is accepted for publication). All listed specimens designated by a field collection number where tail tips or toes clips collected during field surveys by Environment Australia (designated by ''C'', ''W'', or ''M'') or C. Starr (designated by ''CS''). Museum acronyms are AM for the Australian Museum, Sydney, and NMV for Museum Victoria, Melbourne. *Tympanocryptis tetraporaphora*: GenBank#DQ529271, AMR163347, northern NSW; *T. pinguicolla*: GenBank#DQ529254, C1, Kuma Nature Reserve, Cooma, NSW; *T. pinguicolla*: GenBank#DQ529255, C11, Devereanux, Cooma, NSW; *T. pinguicolla*: GenBank#DQ529256, C12, Devereanux, Cooma, NSW; *T. pinguicolla*: GenBank#DQ529257, C2, Devereanux, Cooma, NSW; *T. pinguicolla*: GenBank#DQ529258, C3, Quartz Hill, Cooma, NSW; *T. pinguicolla*: GenBank#DQ529259, C6, Kuma, Cooma, NSW; *T. pinguicolla*: GenBank#DQ529260, C8, Kuma, Cooma, NSW; *T. pinguicolla*: GenBank#DQ529261, C9, Quartz Hill, Cooma, NSW; *T. pinguicolla*: GenBank#DQ529262, W3, Woden, Canberra, Australian Capital Territory; *T. pinguicolla*: GenBank#DQ529263, M2, Majura Firing Range, N Canberra Airport, Australian Capital Territory; *T. pinguicolla*: GenBank#DQ529265, CS1, Mount Tyson, Halford, Queensland; *T. pinguicolla*: GenBank#DQ529266, CS14, Mount Tyson, Halford, Queensland; *T. pinguicolla*: GenBank#DQ529268, CS10, Mount Tyson, Halford, Queensland; *T. pinguicolla*: GenBank#DQ529267, CS33, Bongeen, Woodridge, Queensland; *T. pinguicolla*: GenBank#DQ529269, CS41, Bongeen, Woodridge, Queensland; *T. pinguicolla*: GenBank#DQ529270, CS46, Bongeen, Woodridge, Queensland; *T. pinguicolla*: GenBank#DQ529264, NMVD3483, mouth of the Yarra River, Melbourne, Victoria (specimen dates from 1906).

Our analyses also included the following previously published sequences (Melville et al. 2001; Schulte et al. 2003): *Rankinia diemensis*, AF375619; *Pogona vitticeps*, SAMA R42415, AY133026, Mabel Creek Station, South Australia; *Tympanocryptis centralis*, SAMA R31761, AY133030 208 km E Pipilyatjarra, South Australia; *Tympanocryptis cephalus*, SAMA R22854, AY133027, 21 km N Roebourne, Western Australia; *Tympanocryptis houstoni*, WAM R119738, AY133028, 40 km W Mundrabilla Roadhouse, Western Australia; *Tympanocryptis intima*, SAMA R40665, AY133029, Moolawatana Homestead, South Australia; *Tympanocryptis lineata*, AF128475; *Tympanocryptis pinguicolla*, ANWC R5786, AY133031, Majura Firing Range, N Canberra Airport, Australian Capital Territory; *Tympanocryptis tetraporophora*, ANWC R5612, AY133032, Morella Station, 65 km NNW of Walgett, New South Wales.

Samples for morphological analysis

Museum acronyms are AM for the Australian Museum, Sydney, ANWC for the Australian Wildlife Collection CSIRO, Canberra, and QM for the Queensland Museum, Brisbane. Seven specimens were from the Darling Downs, QLD (Bongeen: QMJ81871, QMJ81870, QMJ81784, QMJ34744; Brookstead: QMJ82087, QMJ82088; Dalby: AMR16957) and eleven from Canberra/NSW (Bathurst NSW: AMR26077, AMR25980;

Woden station, Canberra: ANWCR05656, ANWCR05657, ANWCR05658, ANWCR05789; and Canberra Airport ACT: ANWCR05659, ANWCR05785, ANWCR05786, ANWCR05787, ANWCR05788).

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