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Conservation genetics of the endangered grassland earless dragon *Tympanocryptis pinguicolla* (Reptilia: Agamidae) in Southeastern Australia

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Abstract

The grassland earless dragon, *Tympanocryptis pinguicolla*, is listed as endangered throughout its range. A recent taxonomic study based on both morphological and allozyme data elevated *pinguicolla* from a subspecies of *T. lineata* to full species status, but the allozyme data showed considerable differentiation among *pinguicolla* populations. To investigate the magnitude and nature of these differences with an independent data set, we targeted key *pinguicolla* populations and sequenced an approximately 900 base pair DNA fragment of the mitochondrial genome that includes half of the ND4 gene and three tRNA genes. We obtained sequence data from 21 individuals drawn from the three *T. pinguicolla* populations, included representatives of two other *Tympanocryptis* species and used an *Amphibolurus* species as an outgroup. Seven mitochondrial haplotypes were found among the 21 *T. pinguicolla* samples – two in the Australian Capital Territory (ACT) and five in the Cooma region, Phylogenetic signal in the data sets was extremely strong and a variety of phylogenetic analyses of the data all resulted in the same single fully resolved tree. There are 37 unique differences in the ND4 gene between the ACT and Cooma populations. This translates into genetic differences of between 5.76% and 6.23% between the two populations. In comparison to studies on other reptile groups in which the same fragment of DNA was used, the differences found between the ACT and Cooma populations are more in line with species-level differences than differences within a single species and suggests that these populations should be considered separate taxonomic units.

Introduction

The agamid lizard genus *Tympanocryptis* comprises eight species ranging across much of Australia. These species occupy a variety of habitats and most are relatively common and abundant (Cogger 2000). This also is true of four of the five subspecies of *T. lineata*, but *T. lineata pinguicolla* is an extreme habitat specialist, living only in grasslands and using spider burrows as retreat sites (W. Smith, L. Nelson, pers. com.). As much of the southeastern region of Australia is now under pasture, this lizard's habitat is greatly diminished. *Tympanocryptis pinguicolla* is now formally listed as endangered throughout its range with legislation protecting it in the Australian Capital Territory, New South Wales, Queensland and Victoria (reviewed in Smith et al. 1999).

In a recent study, Smith et al. (1999) used a combination of detailed morphometric and allozyme electrophoresis data to examine the status of *pinguicolla* in relation to other *Tympanocryptis* species. Their sampling included specimens throughout the known range of the species, and also included specimens from the Australian Capital Territory (ACT) as well as populations south of Canberra in the Cooma region. These localities are important because these lizards occupy only two known localities in the ACT (which has its own independent wildlife legislation), one of which is the airport. These ACT populations are seperated by approximately 15 km. Smith et al.

(1999) concluded that *pinguicolla* was distinct from other *T. lineata* and raised *pinguicolla* to a full species (Smith et al. 1999). Their results are compelling in that *pinguicolla* clearly falls out as a distinct taxon in all morphometric analyses and it also displays fixed allozyme differences from all other *Tympanocryptis* taxa.

However, Smith et al. (1999) also found considerable allozyme heterogeneity *among* the *pinguicolla* samples in six allozyme loci (their Table 3). The ACT and Cooma samples were particularly divergent, suggesting possible genetic substructuring. However, their allozyme data set was small and so the authors were not able to consider these difference further except to comment that "although sample sizes are still small at each of the four locations, the pattern across all six loci suggests that the populations are not panmictic." Here we use an independent molecular data set to test the hypothesis that *Tympanocryptis pinguicolla* is distinct from other *T. lineata* subspecies and that populations display significant levels of population substructure and may in fact represent separate taxonomic units.

Materials and methods

Samples

A total of 21 *Tympanocryptis pinguicolla* samples were processed: four from each of the two populations from the ACT (Majura and "Woden") and thirteen from a population near Cooma, approximately 150 km south of the ACT (Table 1). The maximum distance between collection sites within the Cooma area was approximately 20 km, but the area is essentially continuous habitat. At present, no populations between the ACT and Cooma have been found. The samples in this study comprised toe clips obtained from wild animals as part of an ongoing study of the lizard's ecology and conservation. We included a single representative each of *T. lineata centralis*, *T. lineata houstoni*, and *T. lineata lineata* to examine their relationship to *T. pinguicolla*. We were not able to obtain tissue from the only other *T. lineata* subspecies, *T. l. macra*. We included the agamid lizard *Amphibolurus muricatus* as the outgroup.

DNA and PCR

Total genomic DNA was extracted using a modified Hexadecyl-trimethyl-ammoniumbromide (CTAB) protocol. Approximately 100 mg of genomic DNA was used as template for PCR amplification experiments. For each sample we targeted an approximately 900 base pair (bp) DNA fragment of the mitochondrial genome which included the 3′ half of the ND4 gene and most of the tRNA cluster containing the Histidine, Serine and Leucine tRNA genes. This region was targeted because work at comparable taxonomic levels in other squamate reptile groups indicated useful levels of variability (Kraus et al. 1996; Benabib et al. 1997; Forstner et al. 1998).

Primers to amplify this region were selected after compiling a general lizard ND4 alignment from sequences deposited on GENBANK. No agamid lizard ND4 sequences were available for comparison so a suite of sequences from other diverse lizard groups was evaluated and assessed for conserved regions appropriate for primer design. The target fragment was amplified using modified primers ND4 and Leu (Arévalo et al. 1994). Reactions were 40 μl in volume and contained 10 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.25 mM dNTPs and 2 units of *Taq*-polymerase (*Amplitaq* DNA polymerase, Perkin-Elmer). PCR amplification of double-stranded product was done using a Corbett PC-960C cooled thermal cycler using a step-down cycling profile. Reactions were initially denatured at 94 °C for 5 min., followed by an annealing step at 65 °C for 30 s and extension at 72 °C for 1.5 min. This was followed by a further round of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and extension at 72 °C for 1.5 min. The annealing temperature was then dropped by 5 °C in the next 2 rounds of cycling. This "stepping-down" in annealing temperature was repeated until a final annealing temperature of 40 °C was reached. The next 30 cycles then were performed with this annealing temperature. A final extension step at 72 °C was done for 7 min.

DNA sequencing

PCR products were gel purified using the BRESA-clean kit (Geneworks) following manufacturer's instructions. Following purification, products were directly sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit. Reactions were done using half the amount of Ready Reac-

Table 1. Summary of specimens sampled and their locality information. *Amphibolurus muricatus* was used as the outgroup in phylogenetic analyses. Due to their conservation status, all *Tympanocryptis pinguicolla* used in this study were supplied in the form of toe clips from an ongoing study of the ecology of these populations by the ACT Environment Australia, thus these animals were not killed. ANWC = Australian National Wildlife Collection, CSIRO. SAM = South Australian Museum. The haplotype identification number corresponds to those in Table 2 and Figure 1. The localities for Cooma samples of *T. pinguicolla* are all within 20 km of each other and essentially continuous habitat.

Species	Museum Number	Haplotype ID	Locality
Amphibolurus muricatus			Canberra, Australian Capital Territory
Tympanocryptis diemensis	ANWC R5629		Badger Corner, W of Lady Barron, Flinders Island, Tasmania
Tympanocryptis lineata centralis	SAM R28288		Everard Park, South Australia
Tympanocryptis lineata houstoni	SAM R22986		14k west Cocklebiddy, Western Australia
Tympanocryptis lineata lineata	SAM R32890		Wanna, South Australia
Tympanocryptis pinguicolla (M1)		2	"Majura", Australian Capital Territory
Tympanocryptis pinguicolla (M2)		1	"Majura", Australian Capital Territory
Tympanocryptis pinguicolla (M3)		2	"Majura", Australian Capital Territory
Tympanocryptis pinguicolla (M4)		1	"Majura", Australian Capital Territory
Tympanocryptis pinguicolla (W1)		1	"Woden", Australian Capital Territory
Tympanocryptis pinguicolla (W2)		1	"Woden", Australian Capital Territory
Tympanocryptis pinguicolla (W3)		1	"Woden", Australian Capital Territory
Tympanocryptis pinguicolla (W4)		1	"Woden", Australian Capital Territory
Tympanocryptis pinguicolla (C1)		3	Kuma Nature Reserve, Cooma, New South Wales
Tympanocryptis pinguicolla (C2)		7	Devereanux, Cooma, New South Wales
Tympanocryptis pinguicolla (C3)		4	Quartz Hill, Cooma, New South Wales
Tympanocryptis pinguicolla (C4)		7	Devereanux, Cooma, New South Wales
Tympanocryptis pinguicolla (C5)		3	Quartz Hill, Cooma, New South Wales
Tympanocryptis pinguicolla (C6)		5	Kuma, Cooma, New South Wales
Tympanocryptis pinguicolla (C7)		3	Kuma/Devereanux, Cooma, New South Wales
Tympanocryptis pinguicolla (C8)		3	Kuma, Cooma, New South Wales
Tympanocryptis pinguicolla (C9)		6	Quartz Hill, Cooma, New South Wales
Tympanocryptis pinguicolla (C10)		3	Quartz Hill, Cooma, New South Wales
Tympanocryptis pinguicolla (C11)		7	Devereanux, Cooma, New South Wales
Tympanocryptis pinguicolla (C12)		3	Devereanux, Cooma, New South Wales
Tympanocryptis pinguicolla (C13)		7	Devereanux, Cooma, New South Wales

tion Premix and 1.6 pmol of each amplification primer. Approximately 10–15 ng of purified PCR product was used as template. Cycle sequencing was done using either of the following profiles for 30 cycles: 1) 96 °C for 30 s, 50 °C for 15 s, 60 °C for 4 min, or 2) 96 °C for 30 s, 60 °C for 4 min. Ramping was set for 1 °C/second. On completion of cycle 30 reactions were brought to 4 °C. Extension products then were removed from under the oil, placed in 1.5 ml tubes and the volume brought to 20 μl with deionised water. Dried extension products were resuspended in 3–4 μl of loading dye. Sequences were electrophoresed on 5.2% denaturing polyacrylamide (PAGE-PLUS, Amresco) gels (36 cm well-to-read) and analysed on the ABI 377XL^{TM} automated DNA sequencer.

Sequence editing and analysis

Sequence data were edited using Sequencher 3.0 (Gene Codes Corporation), and provisionally aligned using the default parameters of ClustalX (Thompson et al. 1994). Following elimination of alignment gaps, aligned sequences then were translated into amino acid sequences using the vertebrate mitochondrial genetic code. This was done to determine if these data were truly mitochondrial in origin. No premature stop codons were observed therefore we conclude that all sequences obtained are true mitochondrial copies.

Phylogenetic analyses

We employed the three most frequently used phylogenetic analyses to assess any possible effect on

the topology due to analysis type alone. Parsimony, neighbour-joining and maximum likelihood analyses were performed using PAUP* Version 4.0 (Swofford 2000). Data analysed using parsimony criteria were weighted in a variety of ways: unweighted, or a ti/tv ratio of 2, 5, 10 or transversion only were used. The actual ti/tv ratio was estimated from the data via maximum likelihood. The ti/tv ratios used for parsimony analyses were designed to approximate and flank the actual ratio. Neighbor-joining distance analyses used Jukes-Cantor (1969) genetic distances. Maximum likelihood analyses were performed on haplotypes only and the ratio of transitions to transversions, as well as evolutionary rates at first and third codon positions (relative to second positions) were estimated iteratively until the likelihood of the topology had been maximised. A total of 1000 bootstrap pseudoreplicates were performed in the parsimony analysis to examine the relative support for each branch.

Genetic distances were calculated using the K2, K3, Tamura-Nei and Maximum Likelihood substitution models for the following data sets: 1) all data (813 nbp), all 21 samples, 2) ND4 data (700 nbp), all 21 samples, 3) all data, seven haplotype samples, and 4) ND4 data, seven haplotype samples. All distance estimates showed the same patterns of genetic diversity.

Results

The edited alignment is 813 nbp in length, comprising 700 nbp of the 3′ end of ND4 and 113 nbp of the HSL tRNA cluster, including complete sequences of tRNAs Histidine and Serine and partial sequence of tRNA-Leucine. The alignment commences at a third codon position. A total of 205 sites were variable and 87 informative under parsimony, all of which occurred within the ND4 region. Only minor differences were found within either the ACT of Cooma populations. Two mitochondrial haplotypes were detected among the eight ACT samples while five different mitochondrial haplotypes were detected among the thirteen Cooma samples (Table 1 & 2). A total of 37 substitutions are diagnostic between the ACT and Cooma populations. Jukes-Cantor (1969) interspecific genetic distances among the haplotypes are presented in Table 2. The distributions of 10,000 randomly generated trees were left-skewed ($g_1 = –0.447$, $p < 0.01$) indicating strong phylogenetic signal in the data for parsimony analyses (Hillis 1991; Hillis and Huelsenbeck 1992).

The greatest proportion of substitutions occurred at third codon positions. Thirty-three (75%) of all substitutions among *T. pinguicolla* samples occurred at third positions, of which 30 (90.1%) were diagnostic between the ACT and Cooma populations. Those three substitutions which were not diagnostic were all A ↔ C transversions detected at third positions. These were the only transversions observed in the entire data.

Ten substitutions (22.7% of total substitutions) occurred at first positions, of which seven (70%) discriminate between ACT and Cooma samples. All substitutions at first positions are transitions, only one of which is A ↔ G. One replacement substitution (site 632 in alignment) was detected in the inferred amino acid sequence. However, this change (leucine ↔ phenylalanine) is only diagnostic between the two haplotype groups in the Cooma population (ie. Haplotypes 3 & 4 from 5, 7 & 6). Moreover, this residue replacement is conserved as it occurs between amino acids both having hydrophobic (nonpolar) sidegroups.

A single substitution occurred at a second position (site 384 in alignment). This transition discriminates between ACT and Cooma populations with the presence of a methionine residue in ACT samples contrasting with a threonine residue in sample from Cooma. This represents a radical replacement since methionine has a hydrophobic (nonpolar) sidegroup while threonine possesses a hydrophilic (polar) sidegroup.

Regardless of the type of phylogenetic analysis performed, the same fundamental tree topology was found which separates ACT haplotypes from Cooma haplotypes (Figure 1: for unweighted analysis – length = 317 steps, CI = 0.73, RI = 0.84, RC = 0.61, HI = 0.27). The actual ti/tv ratio calculated by maximum likelihood was 4.89. Figure 1a shows one of two most parsimonious trees generated when the ti/tv ratio was set to 5 in a parsimony analysis. The tree illustrated is identical to that obtained in maximum likelihood and distance analyses. When only transversions are considered, the fundamental difference between the ACT and Cooma is still realised (Figure 1b). As can be seen in Figure 1, bootstrap support is exceptionally high, and in particular we point out the 100% bootstrap value between the ACT and Cooma populations. The trees not only indicate the magnitude of divergence between the ACT and Cooma groups but also indicate subdivisions within both groups. Simulation studies have shown that bootstrap values over 70% can be regarded as particularly strong support (Hillis and Bull 1993).

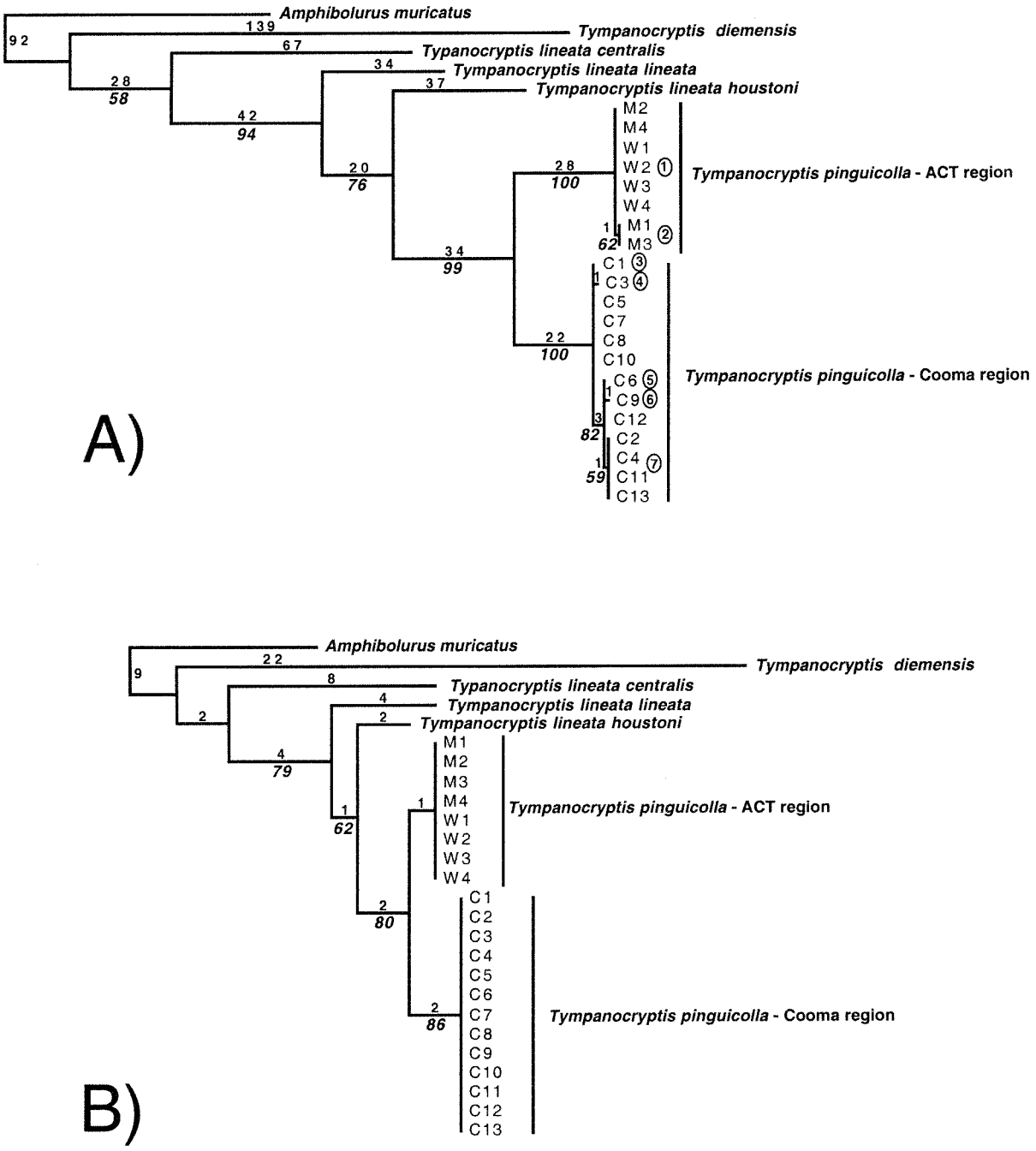

Figure 1. A) One of two most parsimonious trees recovered when transitions are weighted five times more than transversions. This tree is identical to the tree obtained by distance and maximum-likelihood analyses. B) Single most parsimonious tree obtained when transversions only are used. For both trees, numbers above the nodes are branch lengths and numbers below the nodes are bootstrap values.

Table 2. Pair-wise Jukes-Cantor (1969) genetic distances between mitochondrial haplotypes. Haplotype ID numbers for *Tympanocryptis pinguicolla* (1–7) correspond to those used in Table 1 and Figure 1, and abbreviations are used for outgroup species names

	Am	*T.d.*	*T.l.c.*	*T.l.h.*	*T.l.l.*	1	2	3	4	5	6	7
A.m.	–											
T.d.	0.14007	–										
T.l.c.	0.13259	0.14033	–									
T.l.h.	0.10356	0.13882	0.10933	–								
T.l.l.	0.12538	0.14961	0.12250	0.08125	–							
1	0.12720	0.14546	0.13021	0.09414	0.08428	–						
2	0.12869	0.14395	0.12873	0.09556	0.08568	0.00124	–					
3	0.12866	0.15613	0.12281	0.09414	0.07316	0.04869	0.05002	–				
4	0.13013	0.15459	0.12135	0.09554	0.17453	0.05002	0.05135	0.00124	–			
5	0.13163	0.15615	0.12429	0.09839	0.07455	0.05002	0.05135	0.00373	0.00498	–		
6	0.13014	0.15769	0.12282	0.09980	0.07592	0.05135	0.05268	0.00498	0.00623	0.00124	–	
7	0.13016	0.15616	0.12576	0.09698	0.07455	0.05135	0.05268	0.04498	0.00623	0.00124	0.00249	–

Discussion

Our DNA sequence data allows much finer scale understanding of the underlying substructure among the *T. pinguicolla* sample. Analyses of these nucleotide sequence data indicate substantial mitochondrial subdivision within the broader local range of *pinguicolla* and our results corroborate the results of Smith et al. (1999) in regard to *pinguicolla* population substructure.

More specifically to the ACT and Cooma populations, the differences we found between the two populations are substantial. The ACT and Cooma groups differ from one another by at least 5% (range based on ND4 data = 5.76% to 6.23%). Using a conservative mitochondrial calibration of 2% sequence divergence/million years (Brown et al. 1979; Wilson et al. 1985), this suggests that the ACT and Cooma groups have been isolated from one another for approximately 2.5 million years. If we study the results of other studies in which the same gene segment has been used on other reptiles – this 5% divergence would indicate species-level differences.

In a study of the higher level phylogenetics of crotaline snakes (pit vipers) Kraus et al. (1996) obtained ND4 sequenced data for a total of 30 species. In their table of genetic distances they show a range of 5.5%–24.4% sequence divergence between species. Their smallest genetic distance between two distinct species (5.5%) is less than that between the ACT and Cooma populations of *pinguicolla* (5.76% to 6.23%). Kraus et al. did not include subspecies in their study and it is worth noting that the alpha-level taxonomy of pit vipers is well worked out. Thus it is almost certainly not the case that the value of 5.5% in Kraus et al. was due to inadvertently including two members of the same species in their study.

Benabib et al. (1997) used the ND4 gene to study the evolution of viviparity in a group of five North American lizards of the *Sceloporus scalaris* species group. They did not publish a table of genetic distances, but they did publish a table of genetic differences. In the species for which they had the largest number of samples (*Sceloporus bicanthalis*) they found a maximum difference of 30 bp among these samples. The values between species ranged from 40–159. In our study we found a total of 37 differences between the ACT and Cooma population of *T. pinguicolla*. Thus if we are to make a direct comparison between our and the Benabib et al. study, our difference of 37 falls right in between the maximum value for differences within a species and the minimum value between species.

Finally, Forstner et al. (1998) used this gene segment to study the relationships among seven species of North American *Cnemidophorus* lizard. In their table of genetic distances they show a range of 9.2%–30% sequences divergence between species. Their maximum difference found within a species was 2.6%. So again, our values of 5.76% to 6.23% between the ACT and Cooma populations is considerably greater than the maximum difference found within a species in the Forstner et al. study and is more in line with the differences they found between species.

Our results clearly show that the ACT and Cooma populations of *T. pinguicolla* represent unique genetic entities and further that these populations have been separated for a substantial amount of time. In comparison with other groups of reptiles, the genetic difference between the ACT and Cooma populations is in line with species-level differences and is not consistent with differences within a single sub-species.

Within the ACT we have two mitochondrial haplotypes that differ by a single base pair. The sample sizes are very small, but with additional sampling in the ACT region, we may find that there is further substructure within the ACT population. For example, the single base pair difference between the Majura and Woden groups may actually reflect real phylogeographic substructure. We have a better picture of what is going on in Cooma. Sequence divergence estimates among members of the Cooma subsample range from ∼0.1% to 0.75%. This may indicate that there is greater genetic diversity within the Cooma population. This genetic data implies that these lizards have extremely low levels of dispersal, and this is strongly supported by unpublished ecological data (L. Nelson, Pers. Com.).

Tympanocryptis pinguicolla is listed as endangered throughout its range. While our genetic study shows that the ACT and Cooma populations are indeed entirely separate genetic units, it does not follow that one or the other of these populations suddenly does not need protection. As we outlined above, the recent study by Smith et al. (1999) has raised *T. pinguicolla* (including both ACT and Cooma populations) to full species status. Taking their results and our results together, it is probable that the ACT and Cooma populations are still more closely related to each other than either is to other *Tympanocryptis* species. This implies that the geographic distribution of these rare lizards is even more restricted than was previously thought. The phylogenetic substructure within the ACT region and within the Cooma region respectively, also supports this notion. Clearly, both the ACT and Cooma region *T. pinguicolla* are in need of protection and should be considered separate taxonomic units.

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