Limited genetic differentiation among wild *Oryctolagus cuniculus* L. (rabbit) populations in arid eastern Australia

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A systems approach is necessary for effective control of feral rabbit (*Oryctolagus cuniculus* L.) populations in the arid environments of Australia. Localized control procedures may result in local extinctions, but the persistence of the overall population will depend on the probability of recolonization, and hence, the degree of isolation of each local population unit. Genetic markers obtained using allozyme electrophoresis, temperature gradient gel electrophoresis (TGGE) and heteroduplex analysis (HA) were used to characterize the degree of structuring and extent of gene flow among rabbit populations in arid Queensland, Australia. Allozyme allele frequency data showed that there was no significant differentiation among sites (average $F_{ST} = 0.005$) and no isolation-by-distance or environmental discontinuity effects. TGGE/HA results also revealed no significant differentiation in mitochondrial DNA Control Region haplotype frequencies among sites and low interpopulation nucleotide divergence estimates ($N_{ST} = 0.013$). Therefore, rabbit populations exhibited a high degree of gene flow over large geographical areas (1600 km²) and were essentially a single panmictic unit. Unpredictable environmental conditions together with the spatial configuration of habitats which possess different probabilities of extinction may have resulted in repeated local extinctions followed by recolonization and homogenizing gene flow. These data suggest that current rabbit control strategies based on individual warren management may not achieve effective control in arid Queensland.

Keywords: allozymes, control region, genetic differentiation, heteroduplex analysis, mtDNA, temperature gradient gel electrophoresis.

Introduction

Between 13 and 24 wild rabbits (*Oryctolagus cuniculus* L.) of English origin were released in Victoria, Australia in 1859 and within 60 years, rabbits had colonized most of temperate and sub-tropical Australia (Stodart & Parer, 1988). This rapid range expansion can be attributed to efficient reproductive biology, good competitive ability and the absence of significant predators and parasites in Australia (Rural Lands Protection Board, 1987). Pasture damage by rabbits, in conjunction with the loss of grazing through competition for feed is estimated to cost Australia \$A60–90 million annually (Wilson *et al.*, 1992).

Effective population control will depend on the integrity of the local population unit and the degree

of isolation of each unit. It is therefore necessary to determine the appropriate geographical scale for management, i.e. define the boundaries of a demographically independent population or 'management unit' (Moritz, 1994). Despite the high dispersal potential of the rabbit, the warren, a discrete and easily identifiable unit, has been conveniently adopted as the appropriate level at which control procedures should be applied. This level of scale may be appropriate in certain areas of New South Wales. Daly (1979) showed that although the social organization of rabbit populations in areas of New South Wales was hierarchical, it did not result in significant genetic microdifferentiation as paternity was shared and infidelities occurred between social groups. Nonetheless, Richardson (1980) found significant heterogeneity in allele frequencies among warren groups in New South Wales over distances as little as 1 km suggesting that in certain regions of

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Australia control may be effective when applied on a relatively small scale.

Because social organization may differ between localities, it is not possible to generalize the use of warren management techniques (e.g. baiting, fumigation or destruction) to all areas. Furthermore, in arid Australia, control via myxomatosis generally results in only localized control around the inoculation site (warren) as the spread of the virus is limited by the lack of reliable transmission vectors (Rural Lands Protection Board, 1987).

Previous studies in New South Wales provided a preliminary framework on which to base the design of the present study. The dissected dune systems of arid Queensland consist of large parallel sand ridges (1–5 km in length) separated by wide (0.5–5 km in width) claypans (DPI Land Use Report, 1974). In these systems, higher levels of gene flow were expected among local rabbit populations, owing to unpredictable climate and fluctuating rabbit population sizes (Cooke, 1981). Moreover, intergroup interactions are also expected to be more fluid because of the presence of communal grazing areas (claypans) distinct from the breeding areas (sand hills) that support high density rabbit populations. Rowley (1968) and Parer & Parker (1987) have shown that depopulated warrens can rapidly be recolonized by adjacent populations. The present study was designed to investigate differences among rabbit populations on sand hills (with each sand hill consisting of multiple warrens) separated by variable distances and physical geographical barriers in arid Queensland.

In rabbits, up to five polymorphic enzyme systems have been used to detect variation in the underlying genes (Daly, 1979; Richardson, 1980; Richardson *et al.*, 1980, 1986; Webb, 1988). Mitochondrial (mt) DNA has been recognized as a sensitive indicator of population subdivision (Wilson *et al.*, 1985). In particular, the Control Region has a high rate of evolution compared to the rest of the mitochondrial genome (Aquadro & Greenberg, 1983) and permits the greatest opportunity for the detection of genetic differentiation among populations. In the present study, genetic markers were obtained using allozyme electrophoresis, and temperature gradient gel electrophoresis (TGGE) and heteroduplex analysis (HA) of an mtDNA Control Region fragment. TGGE and HA techniques are capable of detecting allelic variation in DNA sequences (see Lessa & Applebaum, 1993) and TGGE can theoretically detect single base changes in genomic DNA (Myers *et al.*, 1988; Riesner *et al.*, 1989; Wartell *et al.*, 1990). The fraction of base mismatches resolved can be

improved by using heteroduplex formation in combination with TGGE (Myers *et al.*, 1985; Myers & Maniatis, 1986). Recently, HA has been applied to haploid loci, where only one type of target sequence is present per sample (e.g. mtDNA; Campbell *et al.*, 1995).

The specific objectives of the present study were (i) to examine genetic differentiation among rabbit populations separated by varying distances (7.5–50 km apart) and thus to determine the relative importance of gene flow and the degree of isolation among local populations, and (ii) to identify potential habitat or geographical barriers to gene flow.

Materials and methods

Sample collection

Approximately 100 adult rabbits were collected from each of nine sites at Bulloo Downs, south-west Queensland (Fig. 1). These sites were selected at varying degrees of geographical isolation, equidistant either side and across the Bulloo River, to allow the examination of distance and possible barrier effects on gene flow. Rabbits were shot and a small section of liver was collected from each individual and stored in a Cryovare vial (Nalgene Co.) under liquid nitrogen. On return to the laboratory, samples were immediately transferred to a -80°C freezer.

Allozyme electrophoresis

Part of each liver sample was homogenized in buffer (according to Richardson *et al.*, 1986) and then centrifuged at 8500 *g* for 15 min at 4°C . The supernatant was removed and used immediately for electrophoresis. Protein electrophoresis was performed on Titan III Zip Zone cellulose acetate plates (Helena Laboratories, TX, U.S.A.), following the running and staining conditions given by Richardson *et al.* (1986). Where multiple loci were present for an individual enzyme, the most anodal was designated as 1. Alleles at polymorphic loci were assigned numerical designations as anodal (1) to cathodal (3). In a previous study on wild rabbits, Richardson *et al.* (1980) screened 26 enzyme loci. An additional 19 enzymes were screened in the present study and two additional polymorphic loci (aldehyde dehydrogenase and aldehyde oxidase) were identified. In total, six polymorphic loci were assayed in this study (*6Pgd-1*, *Est-1*, *Ada-1*, *Ca-1*, *Aldh-1* and *Aox-2*); however, *Ca-1* could not be scored consistently and was later excluded from the analysis.

Fig. 1 Location of rabbit study sites at Bulloo Downs, south-west Queensland.

BIOSYS-1 (Swofford & Selander, 1989) was used to calculate χ^2 contingency tests for homogeneity, Wright's (1951) *F*-statistics and Rogers's genetic distance estimates (Rogers, 1972) from allozyme data.

MtDNA Control Region analyses

Total DNA was extracted from ≈ 100 mg of liver tissue from 10 individuals per site, by grinding to a fine powder in liquid nitrogen and then incubating at 55°C for 3 h in an extraction buffer consisting of 100 mM Tris(hydroxymethyl)aminomethane (Tris), 20 mM EDTA, 100 mM sodium chloride, 10 per cent sodium dodecyl sulphate, 2 M dithiothreitol and 10 mg/mL proteinase K. The isolation procedure consisted of a phenol extraction, followed by a series of phenol:chloroform (1:1) extractions, ending in a chloroform extraction. All centrifugation was performed at 12000 g. DNA was precipitated in 3 M sodium acetate (pH 5.2) and 100 per cent (-20°C) ethanol at -80°C , and then redissolved in 50 μL of Tris-EDTA buffer, pH 7.5.

A 523 bp region of DNA flanking the tRNA-proline gene in the mtDNA Control Region was amplified using polymerase chain reaction (PCR) (Saiki *et al.*, 1988). This portion was amplified using a 22 bp primer created by M. S. Elphinstone (Southern Cross University) of sequence 5'-CTCCACCAT-CAGCACCCAAAGC-3' in the tRNA-proline gene and a 20 bp internal primer of sequence 5'-CCTGA-AGTAGGAACCAGATG-3' created by Meyer *et al.* (1990), located in the central conserved domain of the mammalian Control Region. The equivalent

fragment in other mammalian species has been found to be hypervariable (Saccone *et al.*, 1991).

Individual PCR reactions were performed in a 25 μL volume containing deoxynucleoside triphosphate (Promega) at a final concentration of 100 μM , Boehringer-Mannheim Taq 10 $\times$ buffer (containing magnesium chloride at a final concentration of 1.5 mM), 120 nM of primer 1, 120 nM of primer 2 and 0.5 U of Taq polymerase (Boehringer-Mannheim). The reactions were mixed and overlaid with 25 μL of light paraffin oil. Template DNA (100 ng) was added to the mixture through the oil. Temperature cycling was carried out in a programmable 'Minicycler' thermal controller (MJ Research Inc.) with the following cycle program: (1) 94°C for 30 s, (2) 50°C for 10 s, (3) 72°C for 1 min, (4) 94°C for 10 s, (5) 50°C for 30 s, (6) cycle to step 3, 34 more times, (7) 72°C for 5 min and (8) 4°C for overnight storage. A negative control containing no genomic DNA was included in every PCR experiment. Amplification products and a DNA size marker (ϕ $\times$ 174 - *Hae*III) were resolved in a 1 per cent Tris-borate-EDTA agarose gel, pH 8 at 100 V. The resulting fragment was visualized by ethidium bromide fluorescence on a UV light transilluminator.

PCR products were stored at -20°C until required for heteroduplex analysis by TGGE. The protocol for heteroduplex formation is given in the TGGE Handbook (1993). Each PCR product (≈ 10 ng) was heteroduplexed with a single reference rabbit PCR product.

The DIAGEN horizontal TGGE system was used for heteroduplex analysis of rabbit DNA samples (TGGE Handbook, 1993). Optimal temperature

gradient conditions were determined by electrophoresis (300 V, 20–30 mA, 1.5 h) of the double-stranded product through a 5 per cent polyacrylamide gel, over a perpendicular gradient of temperature from 20 to 60°C. Subsequent electrophoretic runs were of 4 h duration, using a parallel temperature gradient of 11 to 46°C. Internal standards (i.e. known sequence variants of differential mobility) were included on every gel. The DNA was visualized using the silver staining procedure given in the TGGE Handbook (1993).

Approximately 100 ng purified DNA fragment (QIAquick PCR Purification Preps, QIAGEN Inc.) and 3.2 pmol primer were sequenced using ABI (Applied Biosystems) automated DNA sequencing. Each DNA fragment was sequenced (in the majority of cases, twice) from both the 3' and 5' ends. Sequences were aligned by eye using a sequence editor program (ESEE, Version 1.09D). Replicate sequencing (for each haplotype, $n = 5$) was performed, to confirm that individuals of identical haplotype possessed the same sequence.

χ^2 contingency tests were used to determine the significance of haplotype frequencies among sites.

Evolutionary distance among haplotypes was calculated using the Jukes & Cantor (1969) correction and intra- and interpopulation nucleotide divergences were calculated using the distance-based analysis of Lynch & Crease (1990). N_{ST} was calculated according to Lynch & Crease (1990) and N_m values for haploid data were determined according to Hudson *et al.* (1992).

Results

Assessment of genetic differentiation: allozyme markers

Four out of five enzyme loci were highly polymorphic (Table 1). There were no significant differences ($P > 0.05$) in allele frequencies across all sites, for each locus, indicating that alleles occurred at homogeneous frequencies. Sites were grouped on the basis of geographical feature (i.e. north and south of the Bulloo River) and for all loci, there were no significant differences ($P > 0.05$) in frequencies, suggesting that the Bulloo River was not a significant barrier to interchange.

Table 1 Allele frequencies for the nine rabbit populations at Bulloo Downs

Locus	JigBr	MW	Orient	Ponto	ThuBr	Thur1	Thur2	Watts	Willa
<i>6Pgd-1</i>									
<i>n:</i>	100	95	102	100	103	96	106	98	82
1	0.95	0.98	0.95	0.95	0.96	0.95	0.95	0.95	0.98
2	0.05	0.02	0.05	0.05	0.04	0.05	0.05	0.05	0.02
<i>Ada-1</i>									
<i>n:</i>	99	77	84	100	103	71	86	93	80
1	0.22	0.24	0.33	0.25	0.33	0.23	0.24	0.27	0.27
2	0.70	0.68	0.62	0.66	0.59	0.66	0.65	0.60	0.68
3	0.08	0.08	0.05	0.09	0.08	0.11	0.11	0.13	0.05
<i>Est-1</i>									
<i>n:</i>	100	91	102	100	102	96	105	97	82
1	0.52	0.47	0.44	0.44	0.42	0.43	0.44	0.45	0.50
2	0.48	0.53	0.56	0.56	0.58	0.57	0.56	0.55	0.50
<i>Aldh-1</i>									
<i>n:</i>	100	94	101	100	102	96	106	98	82
1	0.61	0.65	0.56	0.60	0.57	0.59	0.58	0.63	0.57
2	0.24	0.20	0.27	0.25	0.25	0.25	0.29	0.19	0.23
3	0.15	0.15	0.17	0.15	0.18	0.16	0.13	0.18	0.20
<i>Aox-2</i>									
<i>n:</i>	100	92	101	100	102	95	105	96	81
1	0.80	0.83	0.80	0.81	0.74	0.75	0.75	0.78	0.75
2	0.20	0.17	0.20	0.19	0.26	0.25	0.25	0.22	0.25

JigBr, Jiggerboo Bore; MW, Molesworth; ThuBr, Thurloo Bore; Thur1, Thurloo 1; Thur2, Thurloo 2; Willa, Willalla.

n, sample size.

Fig. 2 Cluster analysis of rabbit populations using unweighted pair-group average method based on Rogers's genetic distance coefficients.

In the majority of cases (92.5 per cent), loci did not deviate from Hardy–Weinberg equilibrium, with significant deviations ($P < 0.05$) detected in only six out of 45 cases. Significant deviations were not confined to a single locus and were considered to be random.

Rogers's genetic distance estimates based on pairwise comparisons of the nine sites, for the five loci, ranged from 0.014 to 0.066 indicating that populations were very similar to one another. There was no pattern in the clustering of sites relative to position either side of the Bulloo River or with distance among sites (Fig. 2). The linkage distances were so low that any groupings in the cluster could be considered to be random associations.

The absence of any isolation-by-distance effect is supported by a poor correlation ($r = -0.057$, $n = 36$, $P > 0.05$) between genetic distance and geographical distance separating localities.

There was very low (0.5 per cent) genetic differentiation (mean $F_{ST} = 0.005$, range 0.004–0.007) among subpopulations (consistent over all loci), and

consequently, a level of gene flow ($Nm = 52$) that was sufficient to prevent substantial differentiation because of random genetic drift.

Assessment of genetic differentiation: mtDNA markers

Haplotype frequencies are displayed in Table 2. There were no significant differences in haplotype frequencies among all sites ($\chi^2_{16} = 20.03$, $P = 0.2189$) despite the inflation of the χ^2 -value owing to low expected values. There was also no significant difference in haplotype frequencies among sites north and south of the Bulloo River ($\chi^2_2 = 5.18$, $P = 0.075$).

The absolute and percentage sequence divergence among the three haplotypes ranged from 0.8 to 2.2 per cent (Table 3). Replicate haplotypes showed identical sequences. Haplotype frequencies were weighted according to nucleotide divergence among haplotypes, and were used in the calculation of intra- and interpopulation nucleotide divergence. Average intrapopulation diversity was 0.8 per cent, whereas average interpopulation diversity was 0.01 per cent, indicating little or no genetic divergence within and among populations. There were no major associations of sites in the cluster of pairwise interpopulation nucleotide divergence estimates and differentiation among sites was less than that observed in allozyme comparisons.

Table 3 Matrix of pairwise percentage sequence divergence estimates (below diagonal) and absolute distance estimates (above diagonal) for the three rabbit mtDNA haplotypes

Haplotype	A	B	C
A	—	4	7
B	0.78	—	11
C	1.38	2.18	—

Table 2 MtDNA haplotype frequencies for the nine rabbit populations at Bulloo Downs

Locus	JigBr	MW	Orient	Ponto	ThuBr	Thur1	Thur2	Watts	Willa
mtDNA									
<i>n</i> :	10	10	11	12	12	11	11	13	12
A	0.30	0.80	0.73	0.25	0.50	0.54	0.46	0.54	0.67
B	0.40	0.10	0.09	0.33	0.17	0.00	0.09	0.31	0.17
C	0.30	0.10	0.18	0.42	0.33	0.46	0.46	0.15	0.16

JigBr, Jiggerboo Bore; MW, Molesworth; ThuBr, Thurloo Bore; Thur1, Thurloo 1; Thur2, Thurloo 2; Willa, Willalla.

n, sample size.

The correlation between interpopulation nucleotide divergence and geographical distance was extremely poor ($r = -0.035$, $n = 36$, $P > 0.05$) indicating no isolation-by-distance effect.

The N_{ST} estimate indicated that there was little or no (1 per cent) genetic differentiation among subpopulations and an Nm value of 38 indicated a high level of gene flow. Gene flow was therefore the major homogenizing force preventing differentiation because of random genetic drift.

Discussion

In the present study limited genetic microdifferentiation in rabbit subpopulations was shown by the data conforming to Hardy–Weinberg equilibrium and both allozyme and mtDNA analysis indicating little or no genetic divergence and high gene flow within and among populations. There were no trends in divergence that could be attributed to habitat barrier effects or distance effects. The Bulloo River is an ephemeral string of waterholes which only becomes a continuous flow when the channels flood during summer. Therefore, any potential barrier effects to gene flow during summer could be negated by migration in the nonflow periods. These results suggest that in arid Queensland, rabbit populations are effectively panmictic over 1600 km². As there was no isolation-by-distance effect and therefore no local clustering of similar alleles or haplotypes, migrants can be assumed to be drawn at random from a total population comprised of geographically dispersed (sand hill) subpopulations.

The high rate of gene flow observed in this study is contrary to previous investigations in southern Australia that have indicated the minimum distance necessary for significant differentiation to develop among rabbit populations to be 20–30 km based on morphological (skull) divergence (Taylor *et al.*, 1977) and 1 km based on biochemical (allozyme) divergence (Richardson, 1980). At distances comparable to those used in this study, Richardson *et al.* (1980) concluded from allozyme electrophoretic data that southern populations were significantly differentiated and that effective migration was very limited. The different genetic patterns found in the previous and present studies can be attributed to the unique spatial configuration of habitats and resource pressures associated with the arid environment of south-west Queensland resulting in a high level of gene flow between local populations.

Several models have been proposed to explain population and genetic structure in small mammal populations that emphasize the importance of social

structure. The propensity for small mammals, particularly rodents, to form small breeding groups or demes as a consequence of social organization was recognized by Anderson (1970) and Selander (1970). Anderson (1970) concluded that gene flow was so restricted that genetic homogeneity was not maintained among different populations of the same species. Similarly, significant genetic heterogeneity in *Mus* populations was detected over an area as small as a single barn, and was considered to be the result of social structuring (Selander, 1970). This model of demic organization has also been applied to *Peromyscus* (Rasmussen, 1970).

Several ecological studies (Myers & Poole, 1959; Cowan, 1987) have reported a high degree of social organization within rabbit populations. In contrast, several genetic studies on rabbit populations (Daly, 1979; Webb, 1988) have revealed that social structuring may not be so strict as to lead to genetic structuring. In marmot (*Marmota flaviventris*) populations a similar genetic structure was reported, with a high rate of gene flow occurring into colonies and a *de facto* avoidance of inbreeding, reducing the effects of social organization (Schwartz & Armitage, 1980). Daly (1979) suggested that the demic organization model may overemphasize the importance of small mammal social structure in natural populations and that the extent of genetic structuring will depend on environmental diversity (the presence and spatial configuration of different habitats) and conditions (optimum habitats changing with environmental fluctuations). Similarly, the genetic structure of vole (*Microtus californicus*) populations (Bowen, 1982) and cottontail rabbit (*Sylvilagus floridanus*) populations (Scribner & Chessner, 1993) was not solely the result of social structure, but was also found to be dependent on ecological and demographic parameters. In summary, genetic structure cannot be assumed to be the product of a single factor (e.g. social behaviour), but should be considered to be influenced by a range of factors including the modification of behaviour in response to resource availability.

In the long-term, different social and mating systems may arise as a result of environmental heterogeneity. Cowan & Garson (1985) reported that when nest sites were clumped (commonly on heavy burrowing substrate), both burrows and mates were limiting resources and social groups formed. However, where nest sites were sparsely distributed (on light sandy soil) and food was a major limiting factor, social organization was more relaxed.

Daly (1979) reported that local habitat heterogeneity will influence population structure and

dynamics and that gene flow may be increased in two ways; population expansion after a favourable change in the weather pattern and successful dispersal into recently occupied areas after a population crash. In the unpredictable arid dune systems of Queensland, unreliable weather patterns and the highly dissected spatial pattern of habitats which possess different survival properties may lead to large fluctuations in rabbit population size.

Migration as estimated by the island model, does not take into account extreme population fluctuations, where gene flow may result more from recolonization after local extinctions rather than from a regular pattern of dispersal among populations (Slatkin, 1985). In arid south-west Queensland, this scenario may be quite likely because of the catastrophic environment (leading to drastic fluctuations in population size; Cooke, 1981) and continued attempts at localized control, resulting in repeated local extinction and recolonization of depopulated areas (Rowley, 1968; Myers & Parker, 1975a,b). The combined turnover of many local populations in the study area may result in high gene flow among local populations and, overall, a genetically homogeneous system. Gene flow would also be enhanced by contact among individuals in communal feeding grounds (claypans) between separate breeding areas (sand hills).

Mass emigrations of rabbits over large distances have also been observed when resource (food, water, shelter) shortage develops over a large area, as in a drought year in the Australian arid zone (Myers *et al.*, 1994). At this point, it cannot be ascertained whether gene flow is the product of widespread dispersal and/or the result of a series of local extinction and recolonization events. In either case, the presence of extensive gene flow over large distances suggests that the demic organization model is inappropriate for rabbit populations and an environmental diversity model similar to that of Daly (1979) is more appropriate for rabbit populations in the arid dune systems of Queensland. Consequently, management strategies for rabbit populations in these environments should be directed towards control at a regional level as individual warren management will have little long-term impact.

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