Heredity (2010) 104, 502–512

www.nature.com/hdy

ORIGINAL ARTICLE

Molecular detection of hybridization between sympatric kangaroo species in south-eastern Australia

LE Neaves[1], KR Zenger[2], DW Cooper[3] and MDB Eldridge[4]
[1]*Department of Biological Sciences, Macquarie University, North Ryde, New South Wales, Australia;* [2]*School of Marine and Tropical Biology, James Cook University, Townsville, Queensland, Australia;* [3]*School of Biological, Earth and Environmental Sciences, University of New South Wales, North Ryde, New South Wales, Australia and* [4]*Department of Molecular Genetics, Australian Museum, 6 College St, Sydney, New South Wales, Australia*

Introgressive hybridization has traditionally been regarded as rare in many vertebrate groups, including mammals. Despite a propensity to hybridize in captivity, introgression has rarely been reported between wild sympatric macropodid marsupials. Here we investigate sympatric populations of western (*Macropus fuliginosus*) and eastern (*Macropus giganteus*) grey kangaroos through 12 autosomal microsatellite loci and 626 bp of the hypervariable mitochondrial DNA (mtDNA) control region. *M. fuliginosus* and *M. giganteus* within the region of sympatry corresponded, both genetically and morphologically, to their respective species elsewhere in their distributions. Of the 223 grey kangaroos examined, 7.6% displayed evidence of introgression, although no F1 hybrids were detected. In contrast to captive studies, there was no evidence for unidirectional hybridization in sympatric grey kangaroos. However, a higher portion of *M. giganteus* backcrosses existed within the sample compared with *M. fuliginosus*. Hybridization in grey kangaroos is reflective of occasional breakdowns in species boundaries, occurring throughout the region and potentially associated with variable conditions and dramatic reductions in densities. Such rare hybridization events allow populations to incorporate novel diversity while still retaining species integrity.
Heredity (2010) **104,** 502–512; doi:10.1038/hdy.2009.137; published online 7 October 2009

Keywords: introgressive hybridization; microsatellite; mitochondrial DNA; kangaroos

Introduction

The inability to successfully hybridize and produce fertile offspring forms the basis of the biological species concept (Dobzanhsky, 1937; Mayr, 1940). However, when speciation occurs allopatrically, reproductive isolation may remain incomplete, allowing hybridization and the subsequent introgression of genes to occur upon secondary contact (Dowling and Secor, 1997). Although the evolutionary consequences of hybridization can be deleterious, causing sterility or reduced fertility of offspring or resulting in a loss of genetic integrity and homogenization of gene pools (Rhymer and Simberloff, 1996), in some circumstances it may be beneficial by allowing the incorporation of novel genetic diversity and promoting adaptation to new environments or even speciation (Dowling and Secor, 1997; Martinsen *et al*., 2001; Salzburger *et al*., 2002; Smith *et al*., 2003). Although initially considered rare and usually deleterious among mammals, hybridization has been identified, to varying degrees, in various wild eutherian mammal taxa including cetaceans (Willis *et al*., 2004), seals (Lancaster *et al*., 2006; Kingston and Gwilliam, 2007), canids (Vila *et al*., 2003; Verardi *et al*., 2006), felids (Pierpaoli *et al*., 2003; Lecis *et al*., 2006), leporids (Thulin *et al*., 2006; Melo-Ferreira *et al*., 2007), martens (Kyle *et al*., 2003) and squirrels (Ermakov *et al*., 2006; Spiridonova *et al*., 2006). However, despite many macropodid marsupial species readily hybridizing in captivity (Close and Lowry, 1990), relatively few instances of natural hybridization have been reported in marsupials, with the exception of several parapatric rock-wallaby species (Briscoe *et al*., 1982; Eldridge *et al*., 1991; Eldridge and Close, 1992; Bee and Close, 1993). Therefore, increasing our understanding of the occurrence and frequency of hybridization across various mammalian taxa will provide greater insights into its evolutionary and ecological importance.

Among large macropodids, a putative wild hybrid has only been reported between the eastern grey kangaroo, *Macropus giganteus*, and the western grey kangaroo, *Macropus fuliginosus* (Coulson and Coulson, 2001). Although the origins of these two species lie in the eastern (*M. giganteus*) and western (*M. fuliginosus*) sides of continental Australia (Oliver *et al*., 1979; Mead *et al*., 1985), both species currently occur sympatrically over ~0.68 million km^2 of eastern Australia (Figure 1, Caughley *et al*., 1984). The relative densities of the two species vary across this region, with the densities of each species decreasing towards the extremities of their respective ranges. Despite morphological similarities, numerous species-specific features are present (Caughley

Correspondence: Dr LE Neaves, Department of Biological Sciences, Macquarie University, North Ryde, New South Wales 2109, Australia.
E-mail: lneaves@gmail.com
Received 20 July 2009; revised 9 September 2009; accepted 10 September 2009; published online 7 October 2009

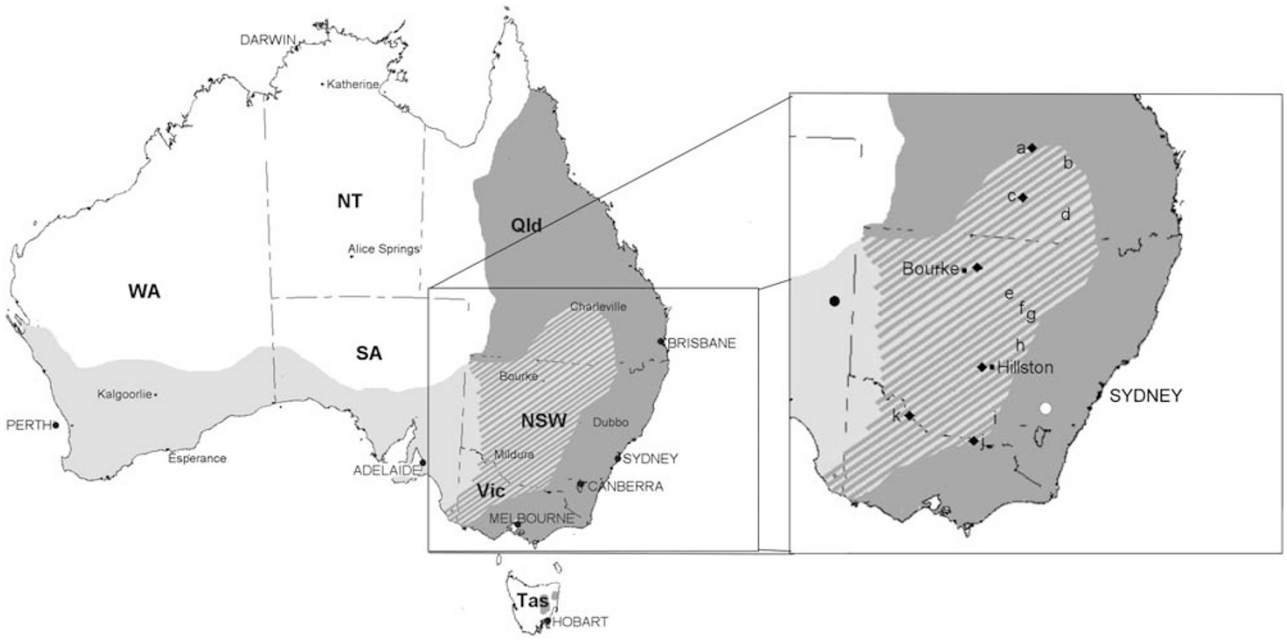

Figure 1 Distribution of eastern (dark shading) and western (light shading) grey kangaroos in Australia, showing their distributional overlap (hatched). The inset shows the zone of sympatry and the areas where samples were collected. The main sampling sites include Hillston ($n=166$) and Bourke ($n=30$). Further grey kangaroo samples were obtained from a: Charleville ($n=3$); b: Mungallala ($n=1$); c: Cunnamulla ($n=2$); d: Bollen ($n=1$); e: Nyngan ($n=3$); f: Mullengudgery ($n=4$); g: Trangie ($n=1$); h: West Wyalong ($n=1$); i: Jerilderie ($n=1$); j: Finley ($n=1$); and k: Hattah Lakes ($n=9$). The sampling sites for representative samples for each species (both $n=20$), located outside the region of sympatry are also shown (black circle = western grey kangaroo sampling site, white circle = eastern grey kangaroo sampling site). Diamonds indicate the sites where hybrid genotypes were located (see text for numbers).

et al., 1984). For example, the pelage of *M. fuliginosus* is darker and more chocolate brown than the grey pelage of *M. giganteus* (for more details, see Coulson and Coulson, 2001). Furthermore, despite some differences in habitat preferences, with *M. giganteus* preferring more open woodland and *M. fuliginosus* denser heath (Coulson, 1990), both species use open grasslands and are often observed in large heterogeneous groups in areas of sympatry (Coulson, 1990, 1999).

Captive-based studies indicate that with no chromosomal differences (Hayman and Martin, 1969; Hayman, 1990), successful hybridization between grey kangaroos may occur despite differences in several reproductive traits such as gestation length and the timing and length of the oestrus cycle (Kirsch and Poole, 1972; Poole and Catling, 1974). However, hybridization appears unidirectional, with the only F1s produced resulting from matings between *M. giganteus* females and *M. fuliginosus* males, the reciprocal never occurred (Poole and Catling, 1974). Of the resultant offspring, F1 females were fertile while males (following Haldane's rule, 1922) appeared sterile, with only primary spermatocytes present in the testes (Poole and Catling, 1974). Female F1 hybrids successfully bred with males of both species, but it was not until the third-generation backcross that spermatogenesis and sperm were present in male offspring (Kirsch and Poole, 1972; Poole and Catling, 1974). F1 hybrids show reproductive and morphological traits intermediate to the parental species, whereas backcrosses were more difficult to distinguish, typically appearing similar to the species to which the hybrid is backcrossed (Kirsch and Poole, 1972; Poole and Catling, 1974).

Previous molecular studies of sympatric grey kangaroo populations detected no evidence of hybridization (Kirsch and Poole, 1972). However, the separation of the species, based on the presence of an unshared polymorphism and no fixed differences at a single locus (*transferrin*), limited even the number of captive bred backcrosses that could be detected (6/14; Kirsch and Poole, 1972). Since then, the sensitivity of molecular genetic and statistical techniques available for investigating introgression have substantially improved (Pritchard *et al*., 2000; Anderson and Thompson, 2002; Manel *et al*., 2005 and references therein); although the accuracy of these techniques differs (Randi and Lucchini, 2002) and an increased likelihood of detecting hybrids potentially generates false positives. However, clustering algorithms such as those used in STRUCTURE (Pritchard *et al*., 2000) and NEWHYBRIDS (Anderson and Thompson, 2002) have explicit predictions for hybrid genotypes, thus minimizing false positives associated with earlier assignment approaches.

Although hybridization of macropodid marsupials in captivity has been relatively well examined, there has been comparatively little examination of hybridization and introgression in wild sympatric populations. Yet, this information could provide insights into the importance of hybridization in marsupial species, as well as in mammals in general. In this study, we examined 12 autosomal microsatellite loci and mitochondrial DNA (mtDNA) to investigate two naturally sympatric species of grey kangaroo. The aim of this study was to ascertain the extent of introgression in these sympatric macropodid populations and test captive study predictions that hybridization is unidirectional.

Materials and methods

Sample collection

Skin, liver or kidney tissue samples were collected from 223 grey kangaroos from across the region of sympatry (Figure 1). Two sites from outside the region of sympatry (~300 km away) were also sampled to provide a representative sample of each species. A total of 20 individuals of *M. fuliginosus* were obtained from South Australia, whereas 20 *M. giganteus* samples were obtained from New South Wales (Figure 1). The morphological species was recorded for all samples, including the representative samples. All samples were adult to sub-adult with no pouch young sampled and were obtained through the commercial kangaroo harvest and from researchers. In the field, samples were preserved in either 20% dimethyl sulfoxide–$NaCl_2$ solution (Kilpatrick, 2002) or 80% ethanol. DNA extraction was performed according to Sigg *et al*. (2005).

Microsatellite amplification and screening

Twelve polymorphic macropodid loci were examined; of them, seven loci were identified in the tammar wallaby (*Macropus eugenii*; T3-1T, T4-2, T19-1, T31-1, T32-1, T46-5 and IL5; Hawken *et al*., 1999; Zenger and Cooper, 2001a), four from the eastern grey kangaroo (*M. giganteus*; G16-1, G20-2, G26-4 and G31-1; Zenger and Cooper, 2001b) and Pa595 from the allied rock wallaby (*Petrogale assimilis*; Spencer *et al*., 1995). Amplification was through PCR in 10 µl reaction volumes containing 100–200 ng of genomic DNA; 2.5 mM $MgCl_2$ (2.0 mM $MgCl_2$ for IL5); 16 mM $(NHa)_2SO_4$; 67 mM Tris HCl (pH 8.8); 0.1% Tween 20; 200 µM of each dCTP, dGTP and dTTP; 20 µM of dATP, 0.05 µl of dATP at 1000 Ci mmol^{-1} (Perkin-Elmer, Glen Waverley, Victoria, Australia); 10 µM of each primer; and 0.5 U of *Taq* polymerase (Bioline, Alexandria, New South Wales, Australia). PCR amplification was carried out in an MJ Research (Watertown, MA, USA) PCT-100 thermocycler, with an initial 94 °C denaturation for 3 min, followed by 'touchdown' cycles of 94 °C denaturation for 30 s, annealing temperatures of 60 °C, decreasing by 2 °C increments at each cycle to 50 °C for 45 s each followed by an extension step of 72 °C for 60 s. On completion of the last touchdown cycle, another 30 cycles with a 50 °C annealing temperature were performed, with a final extension step of 72 °C for 5 min. Amplified products were resolved on a 6% denaturing polyacrylamide gel (Sequa-gel 6; GeneWorks, Hindmarsh, South Australia, Australia), with a standard DNA size reference marker (T7 polymerase; Amersham, Rydalmere, New South Wales, Australia) and visualized by autoradiography according to Taylor *et al*. (1994).

mtDNA amplification and screening

To assess the maternal lineage of an individual, a 626-bp segment of the mtDNA control region, from the tRNA proline gene to the end of the central control conserved region, was amplified using the marsupial-specific primers 15999L (5′-ACCATCACCCAAAGCTGA-3′) and 16498R (5′-CCTGAAGTAGGAACCAGTAG-3′) according to Fumagalli *et al*. (1999). Single-strand conformational polymorphism was performed on amplified PCR products to identify haplotypes and to assess variation (Sunnucks *et al*., 2000). Three separate individuals for each of the unique haplotypes identified were sequenced to ensure the sequence identity of the unique haplotypes. New PCR products in 50 µl reaction volumes and devoid of ^{33}P were amplified as previously described, purified and sequenced using BigDye termination and resolved on an ABI 3730xl automated sequencer (Applied Biosystems, Seocho-Gu, Seoul, Korea; performed at the Macrogen Sequencing Facility, South Korea).

Genetic diversity and differentiation

MICROSATELLITE TOOLKIT EXCEL ADD-IN (Park, 2001) was used to assess both observed and expected heterozygosity (H_o & H_e) and the number of alleles (A) for both representative samples of *M. fuliginosus* and *M. giganteus*. GENEPOP version 3.4 (Raymond and Rousset, 1995) was used to test for linkage disequilibrium and deviations from Hardy–Weinberg equilibrium across all locus population combinations and globally for each of the two representative species groups. The Bonferroni correction was used to adjust the significance levels across multiple tests (Rice, 1989). FSTAT version 2.9.3.2 (Goudet, 2000) was used to estimate the divergence between the two representative species groups and between morphological groups within the sympatric region computing pairwise F_{ST} (Weir and Cockerham, 1984) and R_{ST} (Slatkin, 1995). Differentiation between the two representative species groups was also assessed by analysis of molecular variance (AMOVA) as implemented by GENALEX 6 (Peakall and Smouse, 2006) using F_{ST} and R_{ST}. The number of private alleles, those not shared by the two representative species groups, was determined by rarefaction using the program HPRARE 1.0 (Kalinowski, 2005).

The mtDNA control region sequence was aligned in MEGA 3.1 (Kumar *et al*., 2004) using the Clustal W algorithm (Higgins *et al*., 1994). The number of polymorphic sites (S), nucleotide diversity (π) and the number of pairwise differences (d_A) were determined using MEGA. The extent of sequence differentiation was assessed by AMOVA in Arlequin (Schneider *et al*., 2000), which was also used to calculate the Kimura 2 parameter genetic distance between representative samples of *M. fuliginosus* and *M. giganteus*. To visualize the differences between all the haplotypes identified within the region of sympatry and the representative samples a neighbour joining tree was created in MEGA, with support for the branching topology evaluated using 1000 bootstrap replicates.

Distinguishing hybrid and pure genotypes: simulation study

To determine appropriate cutoff values for distinguishing hybrid and pure genotypes in our study system based on the 12 microsatellites, 500 simulated representative individuals for each species as well as 500 simulated F1 hybrids were generated in HYBRIDLAB version 1.0 (Nielsen *et al*., 2006), using the representative allopatric samples of *M. fuliginosus* and *M. giganteus* as parental populations. Q-values of allopatric and simulated representative *M. fuliginosus* and *M. giganteus* ranged between 0.92 and 0.98 to their respective species clusters in STRUCTURE 2.1 (Pritchard *et al*., 2000), whereas simulated F1 hybrids ranged between 0.3 and 0.7 (see the Results section). On the basis of these ranges

and those of similar studies (Flamand *et al*., 2003; Vaha and Primmer, 2005; Lancaster *et al*., 2006), a threshold of 0.9 was used to distinguish between pure and hybrid individuals in STRUCTURE (that is, <0.9 indicates putative hybrid; separation into hybrid categories was not feasible; see the Results section). In NEWHYBRIDS (Anderson and Thompson, 2002), assignment probabilities of allopatric and simulated representative *M. fuliginosus* and *M. giganteus* to their respective species categories ranged between 0.77 and 1.00, whereas the assignment of simulated F1 hybrids to the F1 category ranged between 0.35 and 1.00. Thus, the category with the highest posterior probability (typically >0.5; Vaha and Primmer, 2005) was assigned to an individual, with definitive assignments indicating probabilities exceeding 0.75. All hybrid categories were considered both separately and collectively when assigning individuals as hybrids in NEWHYBRIDS.

To determine the most appropriate clustering algorithm, NEWHYBRIDS or STRUCTURE, and associated settings, for the detection of backcross hybrids in our study system, the 500 simulated representative individuals for each species and 500 simulated F1 hybrids were used to generate 500 first-, second- and third-generation backcrosses for each species (using HYBRIDLAB; see Supplementary Material). As the frequency of hybridization influences the accuracy of assignment (Vaha and Primmer, 2005), multiple simulated data sets containing variable proportions of hybrid genotypes from each hybrid category were also generated to assess how this variation could influence our results and choice of method (see Supplementary Material).

Detecting hybrids in sympatric grey kangaroos

The results of the simulation study showed the highest proportion of correct assignments occurred in NEWHYBRIDS using a hybrid genotype frequency class (ξ_g) of 0.35 for backcross categories (optimized settings; see Supplementary Material). When the number of simulated hybrids was reduced (that is, rare hybridization; see Supplementary Material), the proportion of correct assignments in optimized NEWHYBRIDS remained higher relative to STRUCTURE and default NEWHYBRIDS (see Supplementary Material). Therefore, as hybridization in our study system was potentially low (Kirsch and Poole, 1972), this method was chosen to examine the empirical microsatellite data set. However, as 5.2% of the simulated first-generation backcrosses were incorrectly assigned to the F1 category using this method, the empirical data set was also examined using both the default NEWHYBRIDS and STRUCTURE to determine the presence or absence of F1 hybrids within the wild sample.

For all data sets, STRUCTURE was run using the following settings: admixed ancestry, correlated allele frequencies, a burn-in period of 50 000 and run length of 10^6, with alpha (α) checked to ensure that burn-in and run lengths were adequate. When estimating K, which was examined from 1–6, $\ln P(X/K)$ (Pritchard *et al*., 2000) was taken into consideration, with five iterations for each value. NEWHYBRIDS was run using both the default (see Anderson and Thompson, 2002) and optimized settings (see Supplementary Material), with a burn-in period of 50 000 and run length of 10^6 iterations. No *a priori* information on individual cluster membership was provided for any analyses, rather the representative allopatric individuals for each species were included in each analysis. Finally, to visualize the similarity between the individuals and putative hybrids from the empirical microsatellite data, a principle component analysis (PCA) was carried out in MVSP version 3.1 (Kovach, 1999), using pairwise genetic distances calculated in GENALEX 6.

Evidence of mtDNA introgression was assessed by comparing the microsatellite genetic identity with the mtDNA haplotype. Hybrid status was assigned where the microsatellite data indicated evidence of introgression at nuclear loci and/or where the species identity indicated by the microsatellite and mtDNA data sets differed, indicating mtDNA introgression. Finally, the genetic identities were compared with the phenotypic data set to assess the correlation between the phenotypic and genetic species within the region of sympatry.

Results

Genetic diversity and differentiation in grey kangaroos

All of the 12 microsatellite loci examined were polymorphic, displaying between 3 and 49 alleles per locus (Table 1). However, T4-2 was found to be monomorphic across all individuals from the geographic range where only *M. fuliginosus* phenotypes were observed (Table 1). There was no evidence of linkage disequilibrium between loci ($P>0.05$) and all loci appear to be in Hardy–Weinberg equilibrium ($P>0.001$) in the representative allopatric samples for each species. The majority of loci displayed unique alleles or marked shifts in allelic frequency (Table 1), with 43 and 78 private alleles (44 and 59%) present in the representative allopatric samples of *M. fuliginosus* and *M. giganteus*, respectively (Table 2).

Six unique mtDNA control region haplotypes were identified in the representative samples for both *M. fuliginosus* (GenBank accession numbers EF555400 and EF555409–EF555413) and *M. giganteus* (GenBank accession numbers AF443127, AF443145–AF443148, AF443154 and AF443162). Overall 95 segregating sites were identified in the 626-bp of mtDNA control region sequence, 32 of which distinguish the two representative species groups. Substantial differences exist between representative samples of *M. giganteus* and *M. fuliginosus*, both in terms of microsatellite ($F_{ST}=0.18$, $R_{ST}=0.53$) and mtDNA ($\Phi_{ST}=0.14\pm0.02$) genetic distances (Figure 2). AMOVA showed the microsatellite variability was partitioned among the two representative species groups ($P=0.0001$) as was mtDNA variation ($P=0.05$). No significant differences in F_{ST} existed between populations inside and outside the region of sympatry for either phenotypic *M. giganteus* ($F_{ST}=0.02$) or *M. fuliginosus* ($F_{ST}=0.05$).

Distinguishing pure and hybrids genotypes in the simulated data set

All representative individuals were correctly assigned to their respective species categories in (default) NEWHYBRIDS, with the assignment probabilities averaging 0.974 (range: 0.802–1.000) and 0.985 (range: 0.845–1.00) in allopatric *M. fuliginosus* and *M. giganteus*, respectively (and ranging between 0.766 and 1.00 in 1000 simulated pure individuals). Thus, individuals were assigned to the

Table 1 Genetic variation in eastern ($n = 20$) and western ($n = 20$) grey kangaroos outside the sympatric zone using 12 microsatellite loci

Locus	*Allelic range*		*No. of alleles*		*Expected heterozygosity*		*Observed heterozygosity*	
	Eastern grey	*Western grey*	*Eastern grey*	*Western grey*	*Eastern grey*	*Western grey*	*Eastern grey*	*Western grey*
G16-1	160–184	146–166	13	9	0.89	0.72	0.79	0.68
G20-2	143–157	147–163	8	9	0.85	0.85	0.89	0.79
G26-4	255–371	227–423	23	26	0.94	0.94	0.86	0.91
G31-1	116–138	120–142	12	12	0.87	0.84	0.88	0.68
T3-1T	149–293	189–245	28	14	0.93	0.91	0.90	0.96
T4-2	120–122	118	2	1	0.19	NA	0.13	NA
T19-1	115–175	147–190	14	11	0.88	0.86	0.89	0.71
T31-1	118–146	110–138	13	10	0.85	0.84	0.76	0.91
T32-1	153–183	147–163	15	9	0.90	0.81	0.82	0.83
T46-5	139–175	131–179	7	10	0.79	0.83	0.70	0.82
IL5	138–172	118–126	14	5	0.79	0.73	0.76	0.56
Pa595	184–256	160–228	16	13	0.93	0.91	0.91	0.76

Abbreviation: NA, not applicable.

Table 2 Genetic diversity indices for microsatellite and mtDNA variation within eastern and western grey kangaroos, outside the zone of sympatry

Genetic marker	*Genetic diversity indices*	*Western grey kangaroo* (Macropus fuliginosus)	*Eastern grey kangaroo* (Macropus giganteus)
Microsatellite	Sample size	20	20
	No. of alleles per locus (± s.d. across loci)	7.83 (± 3.36)	11.00 (± 4.69)
	No. of private alleles (no. of alleles)	43 (98)	78 (132)
	H_e (± s.d. across loci)	0.74 (± 0.07)	0.81 (± 0.06)
	H_o (± s.d. across loci)	0.71 (± 0.03)	0.77 (± 0.03)
	F_{IS}	0.044	0.048
mtDNA	Sample size	20	20
	Haplotypes	6	6
	Polymorphic sites	12	41
	Nucleotide diversity (± s.d.)	0.006 (± 0.002)	0.015 (± 0.002)
	Average pairwise differences	5.4	20

Abbreviations: H_e, expected heterozygosity; H_o, observed heterozygosity; mt DNA, mitochondrial DNA.

category with the highest posterior probability and definitive assignments indicated probabilities exceeding 0.75. The pattern of assignment showed that the vast majority (81.8%) of simulated backcrosses were definitively assigned to a single category using theses values (see Supplementary Material; Supplementary Figure S1). Generations of backcrossing could not be consistently distinguished based on the probability of assignment.

In STRUCTURE, $K = 2$ corresponding to the two morphological/geographical species, with all other values of K deemed highly improbable ($P = 0.9999$; Pritchard *et al*., 2000). Representative allopatric *M. fuliginosus* and *M. giganteus* displayed a high posterior probability of assignment to their respective species groups, with mean values of 0.967 (range: 0.917–0.980) and 0.970 (range: 0.939–0.980), respectively. The same means (with similar ranges) were observed in the 1000 simulated representative individuals. On the basis of the range of Q-values in the representative samples, a threshold of 0.90 was used to distinguish between pure and hybrid individuals in STRUCTURE (i.e. <0.9 indicates putative hybrid). Consistently distinguishing between hybrid categories in STRUCTURE was not possible, because of overlapping Q-values for simulated F1 hybrids (ranging between 0.3 and 0.7, averaging 0.497) and simulated backcrosses (ranging from 0.42 to 0.98, averaging 0.86).

Hybridization in wild sympatric grey kangaroos

Of the 223 kangaroo samples collected from the region of sympatry, 117 were designated as phenotypically *M. giganteus*, whereas 106 were phenotypically *M. fuliginosus*, no instances of ambiguity were noted. These two phenotypic groups largely corresponded to the two genetic clusters evident in the ordination plot (Figure 3). Furthermore, each sympatric genetic/phenotypic group clustered with the representative allopatric samples for their phenotypic species. However, 15 individuals clustered with the opposing phenotypic group to which they were genetically assigned (Figure 3). Although two different groups were apparent within the plot, several individuals were distributed in the intermediate region and potentially represent hybrids (Figure 3).

Analysis of the empirical data through optimised NEWHYBRIDS showed that a total of 17 grey kangaroos exhibited evidence of introgression, although no F1 hybrids were identified (Table 3). Of the 17 grey kangaroos, 14 were identified as *M. giganteus* backcrosses (9 definitively), whereas 3 were identified as *M. fuliginosus* backcrosses (1 definitively; Table 3). Neither STRUCTURE nor NEWHYBRIDS (default) identified additional hybrids (Table 3). Overall, 11 (9 *M. giganteus* and 2 *M. fuliginosus)* of the 17 backcrosses were detected using default NEWHYBRIDS (Table 3). In STRUCTURE, 3 (2 *M. giganteus* and 1 *M. fuliginosus*) of the 17

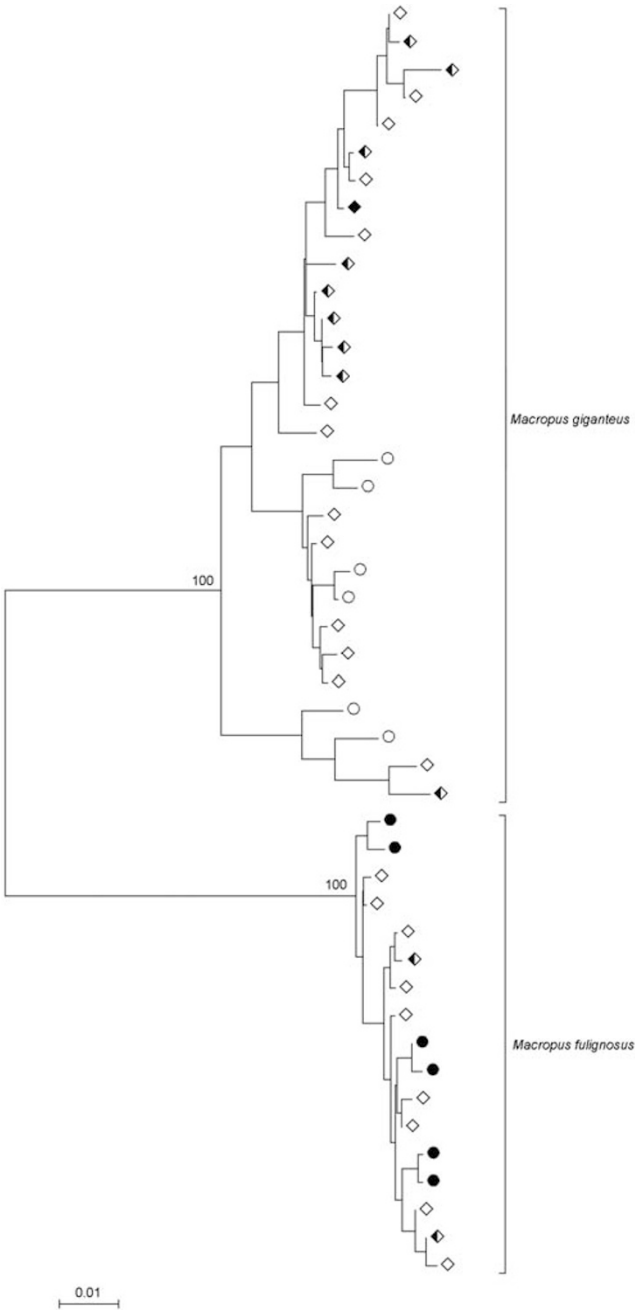

Figure 2 Neighbour-joining tree of grey kangaroo mitochondrial DNA control region haplotypes, showing the differentiation between representative samples of eastern grey (white circles, $n = 20$) and western grey (solid circles, $n = 20$). Haplotypes from the region of sympatry are shown as diamonds. Open diamonds represent pure individuals, whereas hybrids (identified through the clustering algorithms) are represented by closed diamonds. Haplotypes shared by both hybrids and non-hybrids are indicated by half-closed diamonds. Values at nodes indicate statistical support from 1000 bootstrap replicates.

backcrosses were identified as hybrid (<0.90; most likely backcrosses Q-value 0.7–0.9; Table 3). Two of the three hybrids identified by STRUCTURE were also detected using default NEWHYBRIDS. Further examination of the genotypes showed that all the hybrid individuals identified possessed a combination of alleles, which were not shared by the representative allopatric species groups (see Supplementary Table S1). Furthermore, those individuals identified by both programs and/or with a high probability possessed a larger number of potentially introgressed alleles (see Supplementary Table S1). The assignment of the remaining individuals by all three methods was consistent with the phenotypic classification, with the exception of 11 individuals, all previously identified in the PCA (Figure 3). The remaining four individuals identified by the PCA (Figure 3) were identified as *M. giganteus* backcrosses by the clustering algorithms, but classified as *M. fuliginosus* based on phenotype (Table 3). In general, the identified hybrids appear to occur either at the edges of the two clusters or in the intermediate region of the two groups in the PCA (Figure 3). Finally, the representative allopatric samples for both species were assigned to the corresponding phenotypic species from the region of sympatry.

Overall 36 unique mtDNA control region haplotypes were evident among the grey kangaroos within the region of sympatry (GenBank accession numbers EF555400, EF555409–EF555413, EF555426–EF555436, AF443127, AF443145–AF443151, AF443154, AF443162 and EF555437–EF555443). In total, 13 and 23 unique haplotypes were identified among phenotypic *M. fuliginosus* and *M. giganteus*, respectively, and clustered with their respective representative samples (Figure 2). There was no evidence of geographic structuring among haplotypes of either species. Comparisons between the mtDNA and microsatellite data revealed that all except one individual possessed haplotypes consistent with the species or backcross category to which they were genetically assigned (Table 3). The exception, Individual 43, identified as a *M. giganteus* backcross showed evidence of introgressed alleles at both microsatellite loci and mtDNA (Table 3). Several of the identified hybrids shared mtDNA haplotypes, with nine haplotypes identified among the *M. giganteus* backcrosses and two of the three *M. fuliginosus* backcrosses sharing a single haplotype (Table 3).

The geographic location of the putative hybrids was spread across our sampling sites (Figure 1). Not surprisingly, 10 of the hybrids were identified at Hillston, our primary sampling site. Further individuals were located at Cunnamulla ($n = 2$), Charleville ($n = 1$), Bourke ($n = 2$), Finley ($n = 1$) and Hattah Lakes ($n = 1$).

Discussion

A total of 7.6% of grey kangaroos sampled from the region of sympatry displayed evidence of introgression. Although no F1 hybrids were identified, 14 *M. giganteus* backcrosses and 3 *M. fuliginosus* backcrosses were detected. In addition to introgression at nuclear microsatellite loci, a single individual also exhibited introgression of mtDNA. The two phenotypic groups apparent within the region of sympatry corresponded (in 95% of individuals) to the two clusters identified by genetic analyses. Furthermore, the two phenotypic/genetic groups within the region of sympatry corresponded to representative allopatric samples of *M. giganteus* and *M. fuliginosus* from elsewhere in the distribution. Five of the *M. giganteus* backcrosses identified by genetic analyses were classified as *M. fuliginosus* based on overall phenotype. Geographically, hybrids were located throughout the region of sympatry.

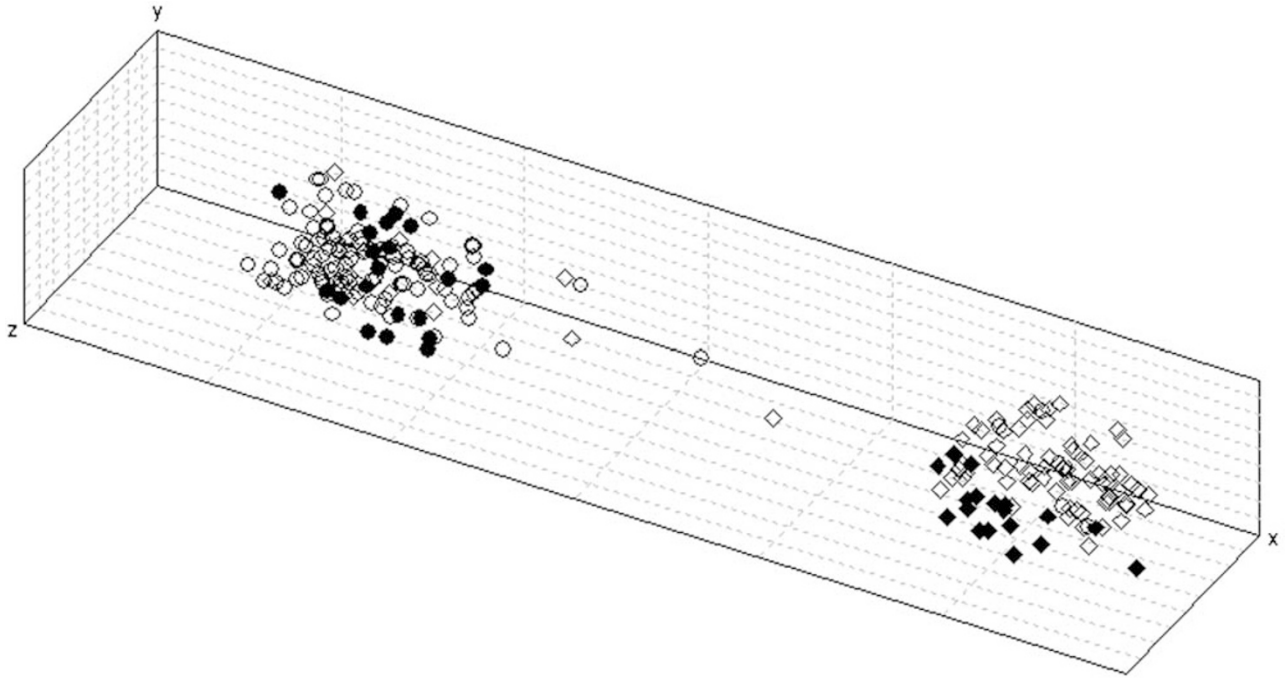

Figure 3 Scores of individual grey kangaroo microsatellite genotypes plotted on the first three axes of a principle component analysis, cumulatively explaining 37.3% of the total genetic variation (27.0, 5.3 and 5.0% on *x*, *y* and *z* axes, respectively). Phenotypically eastern grey (open circles) and western grey (open diamonds) kangaroos from the region of sympatry form two distinct clusters. However, several individuals fall at the outer edge of the clusters or in the space between them. Representative allopatric eastern grey (closed circles) and western grey (closed diamonds) are also shown.

Table 3 The phenotypes, mtDNA haplotypes and assignment probabilities for potential hybrid individuals from the region of sympatry identified either by NEWHYBRIDS or STRUCTURE

Sample ID	*Phenotype*	*mtDNA haplotype*		*NEWHYBRIDS*			*STRUCTURE*	
				Category	*(0.5)*	*(0.35)*	*EGK*	*WGK*
7	WGK	EGK	N24	EGKBX	0.26 (0.27)	0.70 (0.70)	0.97	0.03
9	EGK	?		WGKBX	0.18 (0.37)	0.53 (0.60)	0.11	0.89
23	EGK	EGK	N6	EGKBX	0.64 (0.66)	0.95 (0.96)	0.95	0.05
29	EGK	?		EGKBX	0.88 (0.93)	0.97 (0.98)	0.95	0.05
32	EGK	EGK	N27	EGKBX	0.60 (0.60)	0.96 (0.96)	0.98	0.02
39	EGK	EGK	S27	EGKBX	0.53 (0.53)	0.90 (0.91)	0.98	0.02
43	EGK	WGK	H10	EGKBX	0.14 (0.14)	0.63 (0.63)	0.99	0.01
53	WGK	WGK	H11	WGKBX	0.57 (0.80)	0.87 (0.93)	0.14	0.86
58	WGK	EGK	S28	EGKBX	0.80 (0.85)	0.95 (0.95)	0.92	0.08
59	WGK	EGK	S28	EGKBX	0.80 (0.85)	0.98 (1.00)	0.91	0.09
73	EGK	EGK	S29	EGKBX	0.21 (0.21)	0.70 (0.70)	0.98	0.02
95	WGK	EGK	S30	EGKBX	0.60 (0.63)	0.87 (0.88)	0.96	0.04
107	WGK	WGK	H11	WGKBX	0.35 (0.45)	0.69 (0.72)	0.01	0.99
129	EGK	EGK	S31	EGKBX	0.55 (0.55)	0.88 (0.88)	0.98	0.02
142	EGK	EGK	S29	EGKBX	0.19 (0.20)	0.68 (0.68)	0.97	0.03
154	EGK	EGK	S31	EGKBX	0.49 (0.50)	0.82 (0.83)	0.99	0.01
172	EGK	EGK	S32	EGKBX	0.23 (0.23)	0.55 (0.55)	0.89	0.11

Abbreviations: EGK, eastern grey kangaroo; EGKBX, EGK backcross; mt DNA, mitochondrial DNA; WGK, western grey kangaroo; WGKBX, WGK backcross.
The posterior probabilities to the two groups corresponding to EGK and WGK are shown for STRUCTURE and the probability of assignment to the listed category (that is, EGKBX and WGKBX) using either the default (0.5) or adjusted (0.35) hybrid frequency classes for NEWHYBRIDS. Numbers in brackets indicate the probability when all hybrid categories were considered together. ? indicates mtDNA haplotypes failed to amplify.

The results of this study confirm previous investigations (Kirsch and Poole, 1972) reporting the presence of two morphological groups within the region of sympatry corresponding, both phenotypically and genetically, to *M. fuliginosus* and *M. giganteus* from elsewhere in their respective ranges. *M. giganteus* and *M. fuliginosus* are clearly divergent species with a high percentage of private alleles and significant differences in R_{ST} and F_{ST} reflective of the prolonged period of separation and independent evolutionary histories. Furthermore, the

levels of sequence divergence in the mtDNA control region (0.14 ± 0.02) is similar to that observed among other macropodid species (Eldridge *et al*., 2001).

The results of this study are in contrast with the previous investigations by Kirsch and Poole (1972), which failed to detect evidence of natural hybridization. However, their detection of hybridization was limited as an unshared polymorphism (and no fixed differences) at a single locus (*transferrin*) was the only genetic marker available. This proved insufficient to detect the low level of hybridization identified in this study, and was also limited in its ability to detect known first-generation captive backcrosses (6/14; Kirsch and Poole, 1972). Although, the apparent absence of F1 hybrid genotypes in our data set initially appears paradoxical, similar results have previously been reported (Goodman *et al*., 1999). If hybridization events are rare, then a higher proportion of backcrosses in the population is not unexpected given a single female F1 may produce multiple backcross offspring in her lifetime (Goodman *et al*., 1999). Furthermore, multiple generations may exist within the sample, even though only adults were sampled.

Captive studies in grey kangaroos indicate the presence of several potential pre-zygotic barriers to reproduction between *M. giganteus* and *M. fuliginosus*. Physical differences in the structure of the cloacal eminence as well as the production of species-specific odours by females may allow for species recognition (Kirsch and Poole, 1972). These characteristic differences are potentially among the features that result in the unidirectional hybridization observed in captivity, with male *M. giganteus* frequently failing to recognize female *M. fuliginosus* in oestrus. Therefore, the introgression of mtDNA from *M. giganteus* to *M. fuliginosus* populations could be anticipated as similar cross-species transmission of mtDNA has been reported in other mammals (Lu *et al*., 2001; Ward *et al*., 2001). Yet, we found no evidence of broad-scale introgression of mtDNA, with only a single individual displaying mtDNA introgression,

In contrast to captive predictions, our results indicate natural hybridization occurs in both directions. Furthermore, despite the difficulties observed in captive *M. giganteus* males successfully identifying F1 hybrids and *M. fuliginosus* females in oestrus, a larger proportion of *M. giganteus* backcrosses were identified in the wild. However, captive *M. giganteus* males, successful mated with F1 hybrid females when they were housed with female *M. giganteus* (Kirsch and Poole, 1972), a situation similar to the wild (Arnold *et al*., 1990, 1992). The differences between the predictions based on captivity indicate that, just as captive breeding may prove difficult in some species, the lack of successful matings between *M. fuliginosus* female and *M. giganteus* male kangaroos after 12 years of captive trials (Kirsch and Poole, 1972; Poole and Catling, 1974) does not necessarily preclude the occurrence and potential success of such matings in the wild. Furthermore, breeding and hybridization under captive conditions may be largely dictated by individual preferences. Thus, limitations on the numbers of individuals tested in captive trials may also influence the result, particularly as natural hybridization appears to be a rare event.

Although we have found that species barriers are incomplete in grey kangaroos and secondary contact has resulted in some introgression, the absence of F1 hybrids and the limited introgression detected throughout the region of sympatry indicate the occurrence of occasional genetic leakage rather than a discreet hybrid zone. In grey kangaroos, secondary contact may reinforce species recognition and reproductive barriers, rather than facilitate the homogenization of gene pools or other deleterious consequences. Alternatively, these occasional breakdowns in the species barriers may enable the transfer of novel genetic material, potentially allowing adaptation to new niches, without the loss of a species' genetic integrity. Although the use of 12 microsatellite loci and mtDNA enabled the detection of low-frequency hybridization, a larger number of loci will be required for further investigation into the extent and consequences of low-frequency hybridization. Furthermore, selection against hybrid individuals may mean the frequency of hybridization events is higher than indicated in this study, as only adults were examined and juvenile hybrid offspring may, for various reasons including a reduction in fitness, not have survived to maturity.

The relative densities of the two species appear to vary across the sympatric region, with the densities of each species decreasing towards the extremities of the respective distributions. Our results do not indicate hybrids cluster in a specific geographic region instead hybrids occur throughout the region of sympatry, indicating relative densities are unlikely to significantly influence the frequency of hybridization. However, the variable environmental conditions resulting in fluctuations in density and mate availability in natural populations are another potential factor influence the frequency of hybridization (Seehausen, 2004). The region of grey kangaroo sympatry is prone to fluctuations in rainfall, resulting in dramatic fluctuations in densities of both species, with reductions of up to 40% observed (Caughley *et al*., 1985; Robertson, 1986). Cohabitation on feeding grounds and the formation of heterogeneous groups (Coulson, 1999), coupled with low numbers, may lead to a breakdown in species recognition.

Despite phenotypic similarities, several diagnostic morphological traits, including coloration, distinguish the two species (Caughley *et al*., 1984; Coulson and Coulson, 2001). Within the region of sympatry, these morphological differences allowed the separation of kangaroos into two phenotypic species, which corresponded genetically to *M. giganteus* and *M. fuliginosus*, despite some apparent misclassification (5%). Although captive-bred hybrids typically display intermediate morphological features, backcrosses appeared most similar to the backcrossing species (Kirsch and Poole, 1972). However, although the majority (76%) of sympatric kangaroos genetically identified as backcrosses appeared indistinguishable phenotypically from pure individuals, our results indicate the potential introgression of western grey morphological traits (most likely coloration) into sympatric eastern grey kangaroo populations. On the basis of the results of this study, we suggest that the previously reported putative hybrid (Coulson and Coulson, 2001), which possessed several western grey traits, including facial coloration but was predominately eastern grey in appearance, more likely represents a backcrossed individual, rather than F1. This result highlights the potential for the introgression of other

novel traits, including those associated with reproduction, into *M. giganteus* populations, which have not been examined but may enhanced adaptation to the variable environmental conditions.

Breakdowns in species recognition and subsequent hybridization have been associated with adaptation to new environmental conditions (Dowling and Secor, 1997; Barton, 2001; Seehausen, 2004), particularly where one or both species have recently invaded a new area (Martinsen *et al*., 2001; Salzburger *et al*., 2002). It has been hypothesized that both *M. giganteus* and *M. fuliginosus* colonized the region of sympatry relatively recently (in the last 50 000 years; Oliver *et al*., 1979) and their distributions continue to expand (Shepherd, 1982). As F1 hybrids typically display morphological and reproductive features intermediate of the two species (Kirsch and Poole, 1972), even rare hybridization may allow the introgression of novel genetic material and traits, which could potentially allow a more rapid adaptation to the conditions. Certainly, given *M. fuliginosus* inhabits arid southern Australia, including the Nullarbor Plain, the potential introgression of *M. fuliginosus* genes and associated traits into sympatric *M. giganteus* populations, which inhabit somewhat less arid regions elsewhere in the range, presents interesting possibilities for the potential impacts of even rare hybridization which require continued investigation.

Intriguingly, the breakdown in species recognition between sympatric species of grey kangaroo identified in this study corresponds to hybridization between the usually species-specific nematodes, which parasitize them (Chilton *et al*., 1997). This observation not only highlights the apparent co-speciation of hosts and parasites (Hafner *et al*., 1994; Hafner and Page, 1995) but also suggests that host hybridization may lead to altered host/parasite interactions and the opportunity for genetic exchange among parasites within grey kangaroos from the sympatric region. Further investigation of these observations may provide insights into host/parasite interactions and the history of genetic exchange within the region of sympatry.

Conclusion

In this study, we have shown that recently developed molecular genetic and statistical techniques show the presence of previously undetectable levels of introgression between *M. fuliginosus* and *M. giganteus*. Despite unidirectional hybridization in captivity, a low level of introgressive hybridization in both directions is evident in natural populations, reflective of occasional genetic leakage rather than the presence of a discreet hybrid zone. Introgression was evident throughout the region, and is potentially associated with dramatic reductions in densities resulting from variable environmental conditions. An increased knowledge of the nature and frequency of hybridization is essential to our understanding of the evolutionary processes, as rare hybridization events potentially allow populations to incorporate novel alleles and to maintain diversity while still retaining species integrity.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was carried out with the co-operation of N.S.W DEH under scientific license no. S11250. We have gratefully appreciated the efforts of Ern Snaith, Nicole Payne, Anne Wignall, Ian Beveridge, Taryn Ackland and Dew's Meat for supplying tissue samples. This research was supported by Macquarie University through a Postgraduate Research Fund grant and a Research Awards in Areas and Centres of Excellence scholarship to Linda Neaves. Many thanks to Ian Beveridge, Graeme Coulson, David Briscoe and Adam Stow for discussions, and to Peter Baverstock, Steve Donnellan and Robert Wayne as well as two anonymous referees for comments on an earlier draft of the paper.

References

Anderson EC, Thompson EA (2002). A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* **160**: 1217–1229.

Arnold GW, Steven DE, Grassia A (1990). Associations between individuals and classes in groups of different size in a population of western grey kangaroos, *Macropus fuliginosus*. *Aust Wildl Res* **17**: 551–562.

Arnold GW, Steven DE, Grassia A, Weeldenburg J (1992). Home-range size and fidelity of western grey kangaroos (*Macropus fuliginosus*) living in Remnants of Wandoo Woodland and Adjacent Farmland. *Wildl Res* **19**: 137–143.

Barton NH (2001). The role of hybridization in evolution. *Mol Ecol* **10**: 551–568.

Bee CA, Close RL (1993). Mitochondrial DNA analysis of introgression between adjacent taxa of rock-wallabies *Petrogale* species (Marsupialia: Macropodidae). *Genet Res* **61**: 21–37.

Briscoe DA, Calaby JH, Close RL, Maynes GM, Murtagh CE, Sharman GB (1982). Isolation, introgression and genetic variation in rock wallabies. In: Groves RH and Ride WDL (eds). *Species at Risk: Research in Australia*. Australian Academy of Science: Canberra. pp 73–87.

Caughley G, Grigg GC, Smith L (1985). The effect of drought on kangaroo populations. *J Wildl Manage* **49**: 679–685.

Caughley G, Brown B, Dostine P, Grice D (1984). The grey kangaroo overlap zone. *Aust J Wildl Res* **11**: 1–10.

Chilton NB, Beveridge I, Hoste H, Gasser RB (1997). Evidence for hybridisation between *Paramacropostrongylus iugalis* and *P. typicus* (Nematoda: Strongyloidea) in grey kangaroos, *Macropus fuliginosus* and *M. giganteus*, in a zone of sympatry in eastern Australia. *Int J Parasitol* **31**: 599–602.

Close RL, Lowry PS (1990). Hybrids in marsupial research. *Aust J Zool* **37**: 259–267.

Coulson G (1990). Habitat separation in the grey kangaroos *Macropus giganteus* Shaw and *Macropus fuliginosus* Desmarest Marsupialia Macropodidae in Grampians National Park Western Victoria Australia. *Aust Mammal* **13**: 33–40.

Coulson G (1999). Monospecific and heterospecific grouping and feeding behavior in grey kangaroos and red-necked wallabies. *J Mammal* **80**: 270–282.

Coulson G, Coulson R (2001). A natural grey kangaroo hybrid? *Aust Zool* **31**: 599–602.

Dobzhansky TH (1937). *Genetics and the Origin of Species*. Columbia University Press: New York.

Dowling TE, Secor CL (1997). The role of hybridization and introgression in the diversification of animals. *Annu Rev Ecol Syst* **28**: 593–619.

Eldridge MDB, Close RL (1992). Taxonomy of Rock Wallabies, *Petrogale* (Marsupialia: Macropodidae). I. A Revision of the Eastern *Petrogale* with the Description of Three New Species. *Aust J Zool* **40**: 605–625.

Eldridge MDB, Johnson PG, Close RL (1991). Chromosomal rearrangements in rock wallabies, *Petrogale* (Marsupialia: Macropodidae). IV. G-banding analysis of the *Petrogale lateralis* group. *Aust J Zool* **39**: 621–627.

Eldridge MDB, Wilson ACC, Metcalfe CJ, Dollin AE, Bell JN, Johnson PM *et al*. (2001). Taxonomy of rock-wallabies, *Petrogale* (Marsupialia : Macropodidae). III. Molecular data confirms the species status of the purple-necked rock-wallaby (*Petrogale purpureicollis* Le Souef). *Aust J Zool* **49**: 323–343.

Ermakov O, Surin V, Titov S, Zborovsky S, Formozov N (2006). A search for Y-chromosomal species-specific markers and their use for hybridization analysis in ground squirrels (Spermophilus: Rodentia, sciuridae). *Russ J Genet* **42**: 429–438.

Flamand JRB, Vankan D, Gairhe KP, Duong H, Barker JSF (2003). Genetic identification of wild Asian water buffalo in Nepal. *Anim Conserv* **6**: 265–270.

Fumagalli L, Moritz C, Taberlet P, Friend JA (1999). Mitochondrial DNA sequence variation within the remnant populations of the endangered numbat (Marsupialia: Myrmecobiidae: *Myrmecobius fasciatus*). *Mol Ecol* **8**: 1535–1549.

Goodman SJ, Barton NH, Swanson G, Abernethy K, Pemberton JM (1999). Introgression through rare hybridization: a genetic study of a hybrid zone between red and Sika Deer (Genus *Cervus*) in Argyll Scotland. *Genetics* **152**: 355–371.

Goudet J (2000). FSTAT, a program for Windows (95 and above) to estimate and test gene diversities and Fixation indices (version 2.9.3). Available from http://www.unil.ch/popgen/softwares/fstat.htm.

Hafner MS, Page RDM (1995). Molecular phylogenies and host parasite cospeciation: gophers and lice as a model system. *Philos Trans R Soc Lond B Biol Sci* **349**: 77–83.

Hafner MS, Sudman PD, Villablanca FX, Spradling TA, Demastes JW, Nadler SA (1994). Disparate rates of molecular evolution in cospeciating hosts and parasites. *Science* **265**: 1087–1090.

Haldane JBS (1922). Sex ratio and unisexual sterility in hybrid animals. *J Genet* **12**: 101–109.

Hawken RJ, Maccarone P, Toder R, Marshall Graves JA, Maddox JF (1999). Isolation and characterisation of marsupial IL5 genes. *Immunogenetics* **49**: 942–948.

Hayman DL (1990). Marsupial cytogenetics. *Aust J Zool* **37**: 331–349.

Hayman DL, Martin PG (1969). Cytogenetics of marsupials. In: Benirschke K (ed). *Mammalian Cytogenetics*. Spring-Verlag: New York. pp 191–217.

Higgins DG, Thompson JD, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl Acids Res* **22**: 4673–4680.

Kalinowski ST (2005). HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. *Mol Ecol Notes* **5**: 187–189.

Kilpatrick CW (2002). Noncryogenic preservation of mammalian tissues for DNA extraction: an assessment of storage methods. *Biochem Genet* **40**: 53–62.

Kingston J, Gwilliam J (2007). Hybridization between two sympatrically breeding species of fur seal at Iles Crozet revealed by genetic analysis. *Conserv Genet* **8**: 1133–1145.

Kirsch JAW, Poole WE (1972). Taxonomy and distribution of the grey kangaroos, *Macropus giganteus* Shaw and *Macropus fuliginosus* (Desmarest), and their subspecies (Marsupialia: Macropodidae). *Aust J Zool* **20**: 315–339.

Kovach WL (1999). *MVSP—A Multivariate Statistical Package for Windows, Version 3.1*. Kovach Computing Services: Pentraeth, Wales.

Kumar S, Tamura K, Nei M (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**: 150–163.

Kyle CJ, Davison A, Strobeck C (2003). Genetic structure of European pine martens (*Martes martes*), and evidence for introgression with *M. Americana* in England. *Conserv Genet* **4**: 179–188.

Lancaster ML, Gemmell NJ, Negro S, Goldsworthy S, Sunnucks P (2006). Menage a trois on Macquarie Island: hybridization among three species of fur seal (*Arctocephalus* spp.) following historical population extinction. *Mol Ecol* **15**: 3681–3692.

Lecis R, Pierpaoli M, Biro ZS, Szemethy L, Ragni B, Vercillo F *et al*. (2006). Bayesian analyses of admixture in wild and domestic cats (*Felis silvestris*) using linked microsatellite loci. *Mol Ecol* **15**: 119–131.

Lu G, Basley DJ, Bernatchez L (2001). Contrasting patterns of mitochondrial DNA and microsatellite introgressive hybridisation between lineages of lake whitefish (*Coregonus clupeaformis*); relevance for speciation. *Mol Ecol* **10**: 965–985.

Manel S, Gaggiotti OE, Waples RS (2005). Assignment methods: matching biological questions with appropriate techniques. *TREE* **20**: 136–142.

Martinsen GD, Whitman TG, Turek RJ, Kiem P (2001). Hybrid populations selectively filter gene introgression between species. *Evolution* **55**: 1325–1335.

Mayr E (1940). Speciation phenomena in birds. *Am Nat* **74**: 249–278.

Mead RJ, Moulden DL, Twigg LE (1985). Significance of sulfhydryl compounds in the manifestation of fluoroacetate toxicity to the rat, brush-tailed possum, woylie and western grey kangaroo. *Aust J Biol Sci* **38**: 139–149.

Melo-Ferreira J, Boursot P, Randi E, Kryukov A, Suchentrunk F, Ferrand N *et al*. (2007). The rise and fall of the mountain hare (*Lepus timidus*) during Pleistocene glaciations: expansion and retreat with hybridization in the Iberian Peninsula. *Mol Ecol* **16**: 605–618.

Nielsen EE, Bach LA, Kotlick P (2006). HYBRIDLAB (version 1.0): a program for generating simulated hybrids from population samples. *Mol Ecol Notes* **6**: 971–973.

Oliver AJ, King DR, Mead RJ (1979). Fluoro acetate tolerance a genetic marker in some Australian mammals. *Aust J Zool* **27**: 363–372.

Park SDE (2001). Trypanotolerance in West African cattle and the population genetic effects of selection. PhD. thesis, University of Dublin.

Peakall R, Smouse PE (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes* **6**: 288–295.

Pierpaoli M, Biro ZS, Herrmann M, Hupe K, Fernandes M, Ragni B *et al*. (2003). Genetic distinction of wildcat (*Felis silvestris*) populations in Europe, and hybridization with domestic cats in Hungary. *Mol Ecol* **12**: 2585–2598.

Poole WE, Catling PC (1974). Reproduction in the two species of grey kangaroos, *Macropus giganteus* Shaw and *M. fuliginosus* (Desmarest) I. Sexual maturity and oestrus. *Aust Wildl Res* **22**: 277–302.

Pritchard JK, Stephens M, Donnelly P (2000). Inference of population structure using multilocus genotype data. *Genetics* **155**: 945–959.

Randi E, Lucchini V (2002). Detecting rare introgression of domestic dog genes into wild wolf (*Canis lupus*) populations by Bayesian admixture analyses of microsatellite variation. *Conserv Genet* **3**: 29–43.

Raymond M, Rousset F (1995). GENEPOP: population genetics software for exact tests and ecumenicism. *J Hered* **86**: 248–249. Available from http://kimura.univ-montp2.fr/~rousset/Genepop.htm.

Rhymer JM, Simberloff D (1996). Extinction by hybridisation and introgression. *Annu Rev Ecol Syst* **27**: 83–109.

Rice WR (1989). Analysing tables of statistical tests. *Evolution* **43**: 223–225.

Robertson G (1986). The mortality of kangaroos in drought. *Wildl Res* **13**: 349–354.

Salzburger W, Baric S, Sturmbauer C (2002). Speciation via introgressive hybridisation in East African cichlids? *Mol Ecol* **11**: 619–625.

Schneider S, Roessli D, Excoffier L (2000). *Arlequin: A Software for Population Genetics Data Analysis* (Ver 2.000). Genetics and Biometry Lab, Department of Anthropology, University of Geneva.

Seehausen O (2004). Hybridization and adaptive radiation. *TREE* **19**: 198–207.

Shepherd NC (1982). Extension of the known range of western grey kangaroos, *Macropus fuliginosus*, and eastern grey kangaroos, *M. giganteus*, in New South Wales. *Aust Wildl Res* **9**: 389–391.

Sigg DP, Goldizen AW, Pople AR (2005). The importance of mating system in translocation programs: reproductive success of released male bridled nailtail wallabies. *Biol Conserv* **123**: 289–300.

Slatkin M (1995). A measure of population subdivision based on microsatellite allele frequencies. *Genetics* **139**: 457–462.

Smith PF, Konings A, Kornfield I (2003). Hybrid origin of a cichild population in Lake Malawi: implications for genetic variation and species diversity. *Mol Ecol* **12**: 2497–2504.

Spencer PBS, Odorico DM, Jones SJ, Marsh HD, Miller DJ (1995). Highly variable microsatellites in isolated colonies of the rock-wallaby (*Petrogale assimilis*). *Mol Ecol* **4**: 523–525.

Spiridonova L, Chelomina G, Tsuda K, Yonekawa H, Starikov V (2006). Genetic evidence of extensive introgression of short-tailed ground squirrel genes in a hybridization zone of Spermophilus major and *S. erythrogenys*, inferred from sequencing of the mtDNA cytochrome b gene. *Russ J Genet* **42**: 802–809.

Sunnucks P, Wilson ACC, Beheregarary LB, Zenger KR, French J, Taylor AC (2000). SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. *Mol Ecol* **9**: 1699–1710.

Taylor AC, Sherwin WB, Wayne RK (1994). Genetic variation of microsatellite loci in a bottlenecked species: the northern hairy-nosed wombat *Lasiorhinus krefftii*. *Mol Ecol* **48**: 245–247.

Thulin C-G, Fang M, Averianov AO (2006). Introgression from *Lepus europaeus* to *L. timidus* in Russia revealed by mitochondrial single nucleotide polymorphisms and nuclear microsatellites. *Hereditas* **143**: 68–76.

Vaha J, Primmer CR (2005). Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. *Mol Ecol* **15**: 63–72.

Verardi A, Lucchini V, Randi E (2006). Detecting introgressive hybridization between free-ranging domestic dogs and wild wolves (*Canis lupus*) by admixture linkage disequilibrium analysis. *Mol Ecol* **15**: 2845–2855.

Vila C, Walker C, Sundqvist AK, Flagstad O, Andersone Z, Casulli A *et al.* (2003). Combined use of maternal, paternal and bi-parental genetic markers for the identification of wolf-dog hybrids. *Heredity* **90**: 17–24.

Ward TJ, Skow LC, Gallagher DS, Schnabel RD, Nall CA, Klolenda CE *et al.* (2001). Differential introgression of uniparentally inherited markers in bison populations with hybrid ancestry. *Anim Genet* **32**: 89–91.

Weir BS, Cockerham CC (1984). Estimating F-statistics for the analysis of population structure. *Evolution* **38**: 1358–1370.

Willis PM, Crespi BJ, Dill LM, Baird RW, Hanson MB (2004). Natural hybridization between Dall's porpoises (*Phocoenoides dalli*) and harbour porpoises (*Phocoena phocoena*). *Can J Zool* **82**: 828–834.

Zenger KR, Cooper DW (2001a). Characterisation of 14 macropod microsatellite genetic markers. *Anim Genet* **32**: 166–167.

Zenger KR, Cooper DW (2001b). A set of highly polymorphic microsatellite markers developed for the eastern grey kangaroo (*Macropus giganteus*). *Mol Ecol Notes* **1**: 98–100.

Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)