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Effects of contrasting demographic histories on selection at major histocompatibility complex loci in two sympatric species of tuco-tucos (Rodentia: Ctenomyidae)

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To explore the impact of history on selection and genetic structure at functional loci, we compared patterns of major histocompatibility complex (MHC) variability in two sympatric species of ctenomyid rodents with different demographic backgrounds. Although *Ctenomys talarum* has experienced a stable demographic history, *Ctenomys australis* has undergone a recent demographic expansion. Accordingly, we predicted that MHC allele frequency distributions should be more skewed, differences between coding and noncoding regions should be less pronounced, and evidence of current selection on MHC loci should be reduced in *C. australis* relative to *C. talarum*. To test these predictions, we compared variation at the MHC class II DRB and DQA genes with that at multiple neutral markers, including DQA intron 2, the mitochondrial control region, and 8–12 microsatellite loci. These analyses supported the first two of our predictions but indicated that estimates of selection (based on ω-values) were greater for *C. australis*. Further exploration of these data, however, revealed differences in the time frames over which selection appears to have acted on each species, with evidence of contemporary selection on MHC loci being limited to *C. talarum*. Collectively, these findings indicate that demographic history can substantially influence genetic structure at functional loci and that the effects of history on selection may be temporally complex and dynamic.

ADDITIONAL KEYWORDS: *Ctenomys australis* – *Ctenomys talarum* – expansion – population.

INTRODUCTION

Historical demographic events may contribute significantly to the current genetic structure of a population, including departures from mutation–drift equilibrium. Both historical reductions in population size (e.g. bottlenecks) and rapid colonizations of new habitats may have lasting impacts on the nature and extent of genetic diversity (Templeton, 1980; Barton & Charlesworth, 1984). More specifically, reductions in population size should eliminate low-frequency alleles, thereby generating observed heterozygosities temporarily in excess of those expected under equilibrium conditions (Watterson, 1984; Cornuet & Luikart, 1996). By contrast, population expansions should increase the prevalence of low-frequency alleles (Slatkin & Hudson, 1991; Harpending *et al.*, 1998), resulting in observed heterozygosities that are lower than expected under equilibrium conditions (Watterson, 1984). Thus, these historical events

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produce distinct genetic signatures that can be used to explore the impacts of population history on current genetic structure.

Although multiple studies have examined the impact of demographic history on neutral molecular markers (Banks *et al.*, 2005; Knowles & Richards, 2005), fewer have explored the effects of history on loci that are subject to selection (but see also Miller & Lambert, 2004; Meyer *et al.*, 2006; Bos *et al.*, 2008). Selection necessarily operates within the same demographic framework as neutral evolutionary processes and, consequently, population parameters (current and historical) that impact the balance between genetic drift and gene flow also shape the effects of selection (Nielsen, 2005). Against a given demographic background, selection may act either to increase (i.e. diversifying selection) or decrease (i.e. purifying selection) genetic diversity and hence relationships among selection, genetic drift and gene flow can vary (Harris & Meyer, 2006; Meyer *et al.*, 2006). At the same time, demographic history, including changes in population size, can produce deviations from the neutral expectations similar to those observed under selection (Kreitman, 2000; Otto, 2000; Nielsen, 2005; Nielsen, Hubisz & Torgerson, 2009). As a result, understanding how demographic history has influenced selection, and, in turn, how selection and neutral evolutionary processes have interacted to shape current genetic structure, can be challenging.

The genes of the major histocompatibility complex (MHC) provide a particularly appropriate system for exploring the effects of demographic history on variation at functional loci. MHC genes code for the molecules engaged in identifying and presenting foreign peptides to the immune system (Apanius *et al.*, 1997; Edwards & Hedrick, 1998). The high levels of variability characteristic of these loci are typically attributed to balancing selection (Hedrick & Thomson, 1983; Klein *et al.*, 1993; Garrigan & Hedrick, 2003). At the same time, variability at these genes can be affected by genetic drift, particularly in populations with small effective sizes (Mikko & Andersson, 1995; Seddon & Baverstock, 1999; Seddon & Ellegren, 2002; but see also Aguilar *et al.*, 2004). Most studies that have examined the effects of historical factors on MHC genes have focused on populations known to have undergone severe, relatively recent reductions in size (i.e. bottlenecks), often as a result of anthropogenic causes (Hedrick *et al.*, 1999, 2000; Hoelzel, Stephens & O'Brien, 1999; van der Walt, Nel & Hoelzel, 2001; Lukas *et al.*, 2004; Weber *et al.*, 2004). By comparison, few studies have considered the role of historical factors in shaping selection on and variability at MHC genes in populations that have experienced historical events over longer timescales; but see also Mona *et al.* (2008).

To explore the impact of demographic history on MHC genes in populations that have not been subject to recent anthropogenic disturbance, we compared patterns and levels of MHC variability in two species of tuco-tucos, which are subterranean rodents in the family Ctenomyidae. The talas tuco-tuco (*Ctenomys talarum*, Thomas, 1898) and the sand dune tuco-tuco (*Ctenomys australis*, Rusconi, 1934) occur sympatrically in coastal habitats in eastern Argentina. Ecological comparisons suggest that, although *C. australis* is particularly restricted to friable soils of the coastal sand dunes in this region (Vassallo, 1993), *C. talarum* occupies both inter-dune and inland habitats with harder, more humid and more vegetated soils (Vassallo, 1993). Previous studies indicate that these species are characterized by markedly different demographic histories. Specifically, analyses of mitochondrial DNA (mtDNA) variation suggest that *C. talarum* has experienced a stable demographic history resulting in a genetic structure that is consistent with differentiation under gene flow–drift equilibrium (Mora *et al.*, 2007). By contrast, *C. australis* appears to have undergone a rapid demographic expansion, as demonstrated by a 'star-like' relationship among mtDNA haplotypes and the absence of an association between geographic and genetic distance among populations of this species (Mora *et al.*, 2006). This expansion is considered to have occurred in response to Quaternary sea level changes that increased the availability of the dune habitat in which these animals occur (Mora *et al.*, 2006); because *C. talarum* occupies a wider range of microhabitats, it may have been less affected by fluctuations in sea level and associated changes in habitat availability, thereby retaining a pattern of genetic isolation by distance (Mora *et al.*, 2007).

These different historical scenarios suggest that demography has played a greater role in shaping genetic structure in *C. australis*. Enhanced genetic drift associated with a recent demographic expansion could affect variation and selection at MHC loci in this species in several ways. For example, although balancing selection should act to maintain multiple MHC alleles at approximately similar frequencies, a recent demographic expansion may counter this tendency, resulting in allele frequency distributions that are skewed towards a few very prevalent alleles and, concomitantly, an excess of low frequency alleles (Harpending *et al.*, 1998). As a result, allele frequencies should be less equitably distributed within populations of *C. australis* than in populations of *C. talarum*. At the same time, balancing selection should act to increase differences in variability between MHC loci and neutral nuclear markers (Meyer *et al.*, 2006), whereas strong genetic drift should tend to reduce this distinction. Accordingly, differences in

variability between MHC exons and neutral portions of the genome should be less pronounced within populations of *C. australis*. Finally, balancing selection that operates against a demographic background characterized by strong genetic drift should have less of an effect on MHC loci (Aguilar & Garza, 2006) and, thus, evidence of recent selection on these genes should be reduced in *C. australis*.

To test these predictions, we examined MHC variability in sympatric populations of *C. talarum* and *C. australis* from Necochea, Buenos Aires Province, Argentina. To characterize the effects of selection on these functional genes, we compared variation at exons 2 of the class II DRB and DQA loci with variation at multiple neutral markers, including DQA intron 2, the mtDNA control region, and 8–12 microsatellite loci. These analyses emphasize the need to compare functional and neutral markers to distinguish the effects of selection from those of neutral microevolutionary forces. At the same time, they reveal striking interspecific differences in immunogenetic variability that suggest an important and dynamic role for demographic history in shaping selection on MHC genes.

MATERIAL AND METHODS

Study species and sample collection

One population each of *C. australis* and *C. talarum* was sampled at Necochea (38°33′S, 38°45′W), Buenos Aires Province, Argentina. At this locality, these species co-occur in coastal habitats that are characterized by sandy soils and that are dominated by grasses and herbaceous plants. Although the study species occur in sympatry, they occupy slightly different habitats; although *C. australis* is restricted to sand dunes characterized by minimal vegetative cover, *C. talarum* inhabits more heavily vegetated inter-dune areas and adjacent coastal grasslands (Malizia, Vassallo & Busch, 1991).

In 2002, *C. talarum* were captured and nondestructive tissue samples were collected as part of studies of the genetic structure of this species (Cutrera, Lacey & Busch, 2005; Cutrera & Lacey, 2006). In brief, the animals were live-trapped and tissue for genetic analyses was obtained by removing the distal 1–2 mm of the outer digit of one hind foot, after which each individual was released at the point of capture. All tissue samples were stored in 95% ethanol until analysis. The same procedure was used in 2003 to obtain tissue samples from *C. australis* as part of a study of the demographic history of this species (Mora *et al.*, 2006). Although samples were obtained in successive years, no between-year differences in population density or composition were detected for either study species, providing no evidence that measures of genetic structure were influenced by the year in which animals were captured. Members of both species were trapped within the same 10-km^2 grid, which encompassed relatively undisturbed sand dune and inter-dune grassland habitat (Fig. 1). All field procedures (capture, handling, and release) conformed to institutional and American Society of Mammalogists guidelines (Gannon, Sikes & Animal Care and Use Committee of the American Society of Mammalogists, 2007). Permits for the field work were granted by the Office of Administration of Protected Areas and Conservation of Biodiversity and Natural Resources, Buenos Aires Province.

Analysis of MHC loci

MHC genotypes for *C. talarum* ($N = 30$) were obtained from Cutrera & Lacey (2006), who examined variability at the class II DRB (exon 2) and DQA (exon 2 and intron 2) loci. DRB and DQA genotypes for 34 *C. australis* were generated as part of the study in accordance with the protocols of Cutrera & Lacey (2006). Although Cutrera & Lacey (2007) reported that 34 *C. australis* were genotyped, only the single most common allele per exon (CtauDRB01, CtauDQA01) was used in that study to assess trans-species polymorphism of MHC loci in the genus *Ctenomys*; patterns and levels of MHC variability in *C. australis* are reported here for the first time. For both study species, the DRB and DQA exons were selected for analysis because they are known to contain the peptide-binding regions of the associated MHC molecules, which are the portions of these genes that are typically most subject to balancing selection (Brown *et al.*, 1993; Hughes & Hughes, 1995).

Characterizing variation at MHC exons

For both MHC exons examined, the number of allelic variants and observed heterozygosity (H_O) were determined using ARLEQUIN, version 3.11 (Excoffier, Laval & Schneider, 2005). Sequence-level allelic differences were characterized using DNAsp, version 4.0 (Rozas *et al.*, 2003). Specifically, for each exon we determined number of segregating sites (S), haplotype diversity (H_D, calculated according to Nei, 1987), nucleotide diversity (π; corrected *sensu* Jukes & Cantor, 1969), and mean pairwise number of nucleotide differences between all sequences sampled (k). Because the same parameters were used to characterize variation at the DQA intron and mitochondrial control region (see below), we were able to compare directly patterns and levels of sequence variability at MHC exons and these neutral markers. To depict historical relationships among MHC alleles,

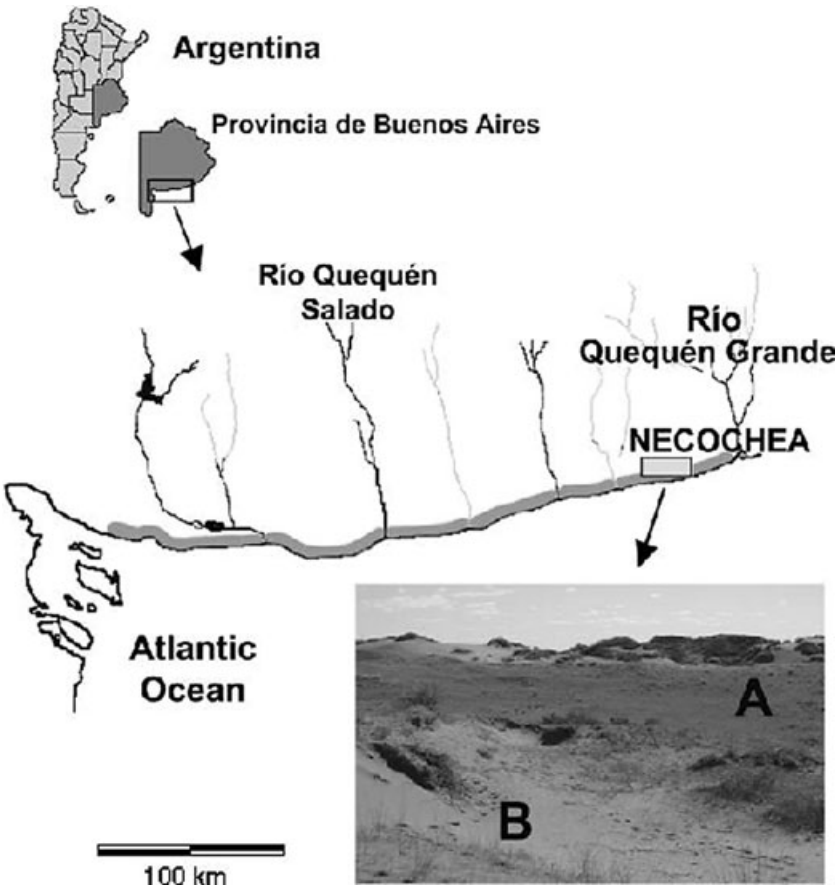

Figure 1. Map of the geographic distribution of *Ctenomys australis* and *Ctenomys talarum* in south-eastern Buenos Aires Province, Argentina, as modified from Mora *et al.* (2006). The area of sympatry is indicated by a grey line along the coast. The primary rivers through this region are indicated, as is the area sampled at Necochea (grey rectangle). The typical habitats of *C. talarum* (A) and *C. australis* (B) are also shown.

minimum spanning trees (MSTs; Excoffier & Smouse, 1994) were generated for each study population using ARLEQUIN, version 3.11 (Excoffier *et al.*, 2005).

Rates of recombination

Traditional methods of inferring phylogenetic and population history typically assume no recombination within loci; violation of this assumption can lead to misinterpretation of historical impacts on genetic variability (Schierup & Hein, 2000; McVean, Awadalla & Fearnhead, 2002). Intragenic recombination (or gene conversion) has been suggested to play an important role in determining variability at MHC loci (Bergström *et al.*, 1998; Schaschl *et al.*, 2006). Although evidence for this process remains equivocal (Martinsohn *et al.*, 1999; Mona *et al.*, 2008), we used the permutation algorithm in LDhat (McVean *et al.*, 2002) to estimate the population recombination parameter ($r = 4N_e r$) for each study population. This analysis is based on a modified version of the composite-likelihood estimation approach of Hudson (2001) that allows for multiple mutations at a site. The population mutation rate parameter ($\theta = 4N_e\mu$) was computed for each population, after which the ratio r/θ was used to estimate the relative effect of recombination and mutation (McVean *et al.*, 2002). To further assess the possibility of recombination within the MHC regions examined, we used DNAsp, version 4.0 (Rozas *et al.*, 2003) to estimate the minimum number of recombination events (Hudson & Kaplan, 1985) per locus required to generate the observed patterns of allelic variability.

Evidence of selection based on substitution rates

Rates of nonsynonymous (d_N) versus synonymous (d_S) basepair substitutions can be used to identify departures from neutrality and to determine the type of selection that has acted on a locus (Kimura, 1983; Ohta, 1993). The nature and strength of selection can

vary among codons (including codons in the same gene; Anisimova, Bielawski & Yhang, 2001) and, thus, we used the maximum likelihood approach of Goldman & Yang (1994) to examine values of ω for the codons within each MHC exon. ω is equivalent to the d_N/d_S selection parameter of Nei & Gojobori (1986) (Goldman & Yang, 1994) and captures information regarding both the type and intensity of selection; for codon-specific analyses, ω is preferred because it allows for variation in the selective pressures experienced by different codons (Nielsen & Yang, 1998). Estimates of ω were generated according to Yang, Wong & Nielsen (2005).

To identify codons subject to positive selection ($\omega > 1.0$) and to determine if the nature of selection varied among codons in the same exon, observed values of ω were compared to seven model distributions of nucleotide substitutions using the CODEML subroutine of PAML, version 3.14 (Yang, 1997). The seven distributions examined were M0 (one-ratio), which assumes the same ω ratio for all codons; M1a (nearly neutral), which assumes both conserved sites (i.e. sites under purifying selection, $0 < \omega < 1$) and selectively neutral sites ($\omega = 1$) among codons for the same protein; M2a (positive selection), which adds a third class of sites with ω as a free parameter (thus allowing for sites with $\omega > 1$); M3 (discrete), which estimates the proportion of conserved, neutral, and unrestricted codons from the data; M7 (beta), which does not allow for positively selected sites ($0 < \omega < 1$); M8 (beta and ω), which adds an additional site class to the beta model to account for sites under positive selection; and M8a (beta + $\omega = 1$), which is similar to model M8, except that the category ω_1 is fixed at $\omega_1 = 1$ (for details on models M1a and M2a, see Yang *et al.*, 2005). The comparison between M8 and M8a has been proposed to be particularly appropriate for detecting positive selection when the beta distribution (M7) provides a poor fit to the true distribution of ω in the interval (0, 1) (Swanson, Nielsen & Yang, 2003). Likelihood ratio tests (Yang *et al.*, 2005) were used to compare model M0 with M3, as well as to compare model M1a with M2a, model M7 with M8, and model M8a with M8. According to Yang *et al.* (2005), when alternative models M2a, M3, and M8 suggest the presence of codons with $\omega > 1$, this can be interpreted as evidence of positive selection. Concomitantly, comparisons of M0 and M3 can be used to detect variable values of ω among codons from the same sequence. Bayesian posterior probabilities were used to identify codons that appeared to be conserved versus neutral or subject to positive selection (Nielsen & Yang, 1998).

Departures from neutrality

To detect departures from neutrality, H–W tests were used to determine whether observed heterozygosities at the DRB and DQA loci were significantly in excess of those expected under equilibrium conditions. Additionally, E–W tests (Ewens, 1972; Watterson, 1986; Garrigan & Hedrick, 2003) were used to determine whether allele frequency distributions were consistent with the effects of balancing selection. Both analyses were conducted using ARLEQUIN, version 3.11 (Excoffier *et al.*, 2005). Because these tests are based on allele frequencies only and do not consider molecular differences between alleles, they are most sensitive to recent events or processes that produce a departure from neutral expectations (Garrigan & Hedrick, 2003).

As an alternative means of detecting departures from neutrality, we calculated Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997) for the MHC exons examined using DNAsp, version 4.0 (Rozas *et al.*, 2003). Because these tests are based on the site frequency spectrum, they are influenced by long-term mutational patterns as well as more recent population dynamics (Garrigan & Hedrick, 2003). Tajima's D uses the normalized difference between θ_W (estimated from the number of segregating sites) and π (mean number of pairwise differences between sequences) to determine whether intermediate-frequency alleles are overrepresented in the population, as would be expected under balancing selection (Tajima, 1989). F_S (Fu, 1997) is based on the probability of observing k or more alleles in a sample of a given size, as estimated from the observed average number of pairwise differences among alleles. To determine the statistical significance of the values of D and F_S obtained, 95% confidence intervals (CI) for these statistics were generated from coalescent simulations conducted in DNAsp, version 4.0 (Rozas *et al.*, 2003). For these analyses, rates of recombination were obtained from LDhat (McVean *et al.*, 2002), as described above, and the genealogy for each locus was generated *sensu* Hudson (1990). For both tests, rejection of the 'standard neutral model' (Rosenberg & Nordborg, 2002) does not imply acceptance of a specific alternative because rejection of the neutral model may occur for multiple reasons, including purely demographic factors (e.g. a population bottleneck) that imitate the effects of balancing selection (Kreitman, 2000).

Analysis of neutral molecular markers

To distinguish between the effects of selection and demography, we assessed variation at multiple neutral molecular markers. Although selection generates a localized signature specific to individual loci, demography is expected to affect the entire genome (Nielsen, 2005; but see also Jensen *et al.*, 2005). As a result, comparisons between functional and neutral

Table 1. Summary of data on microsatellite variation used to assess historical changes in population size in *Ctenomys talarum* and *Ctenomys australis*

Species	N	Number of microsatellite loci used	Mean number of alleles/locus	Mean H_O	Mean H_E
Ctenomys talarum[a]	41	12	6.17 (2.04)	0.57 (0.16)	0.62 (0.17)
Ctenomys australis[b]	50	8	4.56 (1.33)	0.53 (0.22)	0.54 (0.19)

[a]Data are from Cutrera *et al.* (2006).

[b]Data are from Mora (2008).

Eight loci (Soc 1, Soc 2, Soc 5, Soc 6, Soc 8, Hai 3, Hai 4, Hai 11) were amplified in both species, whereas four additional loci (Soc 3, Soc 7, Hai 9, Hai 12) were amplified only in *C. talarum*. N, number of genotyped individuals; H_O, observed heterozygosity; H_E, expected heterozygosity. Standard deviations of mean values are given in parentheses.

markers provide an important opportunity to explore the effects of these distinct determinants of genetic structure (Garrigan & Hedrick, 2003; Thornton *et al.*, 2007). To examine neutral genetic structure in our study populations, we characterized variability at the DQA intron 2; because the DQA intron and exon are closely linked, differences in variability between these regions provide compelling evidence that they are subject to different evolutionary forces. Data on DQA variability in *C. talarum* were obtained from Cutrera & Lacey (2007). Data on DQA variability in *C. australis* were generated as part of the present study. To provide a more comprehensive picture of neutral genetic structure in the study populations and to relate our findings more directly to previous work on these animals, we also examined variation at the control region of the mitochondrial genome, which is not linked to the DRB and DQA loci. Control region data from the same individuals used to characterize MHC variation were obtained from previous studies of genetic structure in *C. talarum* and *C. australis* (Mora *et al.*, 2006; 2007). For both the DQA intron and mtDNA control region, variation was characterized in terms of number of segregating sites (S), H_D, π and k, as described above. To detect potential departures from neutral expectations, Tajima's D (Tajima, 1989) and Fu's F_S (Fu, 1997) tests were conducted for both of these markers using DNAsp, version 4.0 (Rozas *et al.*, 2003).

Data from these neutral markers were also used to quantify apparent historical differences between the study populations. To characterize patterns of differentiation for intron and control region sequences, MSTs were generated using sequence data from the Necochea population of animals examined by Mora *et al.* 2006, 2007). When estimates of Tajima's D and Fu's F_S for these sequences revealed evidence of significant departures from neutrality, the values of these statistics (positive or negative) were used to infer the possible processes underlying these outcomes (Tajima, 1989; Fu, 1997). In addition, historical changes in population size were assessed using LAMARC (Kuhner, 2006), with microsatellite data from *C. australis* (Mora, 2008) and *C. talarum* (Cutrera *et al.*, 2005) serving as an unlinked, neutral nuclear counterpart to the MHC exons. Individuals screened for microsatellite variation (all from Necochea) included those used for MHC and mtDNA sequence analyses (Table 1). For each species, likelihood searches were carried out for both the stepwise mutational model (Kimura & Ohta, 1978) and its Brownian motion approximation (Beerli & Felsenstein, 2001) using a routine where each run consisted of (1) 20 initial chains, each of which sampled 2000 trees at intervals of 20 increments after discarding the 1000 initial trees ('burn-in' period), and (2) five final chains that sampled 10 000 trees at intervals of 20 increments after discarding the 5000 initial trees. This routine was repeated three times using random starting points; the results of these replicates were integrated and checked for convergence across runs and mutational models. This procedure resulted in maximum likelihood estimates and associated confidence intervals for the exponential growth parameter g, which denotes the type and magnitude of potential historical changes in population size.

Two additional methods were used to assess evidence of historical reductions in population size for *C. australis*; neither analysis was considered in previous publications on this species. First, BOTTLENECK (Piry, Luikart & Cornuet, 1999) was used to determine whether observed microsatellite allele frequencies differed significantly from the L-shaped frequency distribution (predominance of low-frequency alleles) expected for non-bottlenecked populations under the stepwise mutation model. Second, the number of microsatellite loci for which the expected heterozygosity under H–W equilibrium (H_E) was greater than the expected heterozygosity estimated from mutation–drift equilibrium conditions (H_{EQ}) was assessed. This analysis is based on the assumption

that, in non-bottlenecked populations, approximately 50% of loci should exhibit excess heterozygosity ($H_E > H_{EQ}$), with the remaining 50% exhibiting insufficient heterozygosity ($H_E < H_{EQ}$) (Cornuet & Luikart, 1996). Significant departures from this expectation were identified using the Wilcoxon signed rank test as implemented by BOTTLENECK (Piry *et al.*, 1999). The results of these analyses were compared with the outcomes of the same tests when applied to *C. talarum* (Cutrera, Lacey & Busch, 2006).

RESULTS

All DNA samples from *C. australis* produced a single, clearly resolved polymerase chain reaction (PCR) product when amplified using primers for either DRB or DQA. No evidence of the presence of chimeric PCR products was detected. After cloning, no more than two sequences per individual were detected at either locus, suggesting that only a single copy of each gene was amplified. No insertions or deletions were detected and, when translated, no stop codons were found in the MHC exons examined. Sequences from DRB exon 2 and DQA exon 2 were compared with those of another well-characterized caviomorph rodent, *Cavia porcellus* (UCSC Genome Browser; http://genome.ucsc.edu). For DRB, the sequence with the highest identity score (75.3%) corresponded to the DRB locus from *C. porcellus*; when these sequences were aligned, no gaps or insertions were identified. Similar results were obtained for DQA exon 2, with 89.9% identity between species. Collectively, these findings suggest that our data on MHC variation in *C. australis* were not confounded by the presence of pseudogenes.

Analysis of MHC variability

GenBank accession numbers for two MHC alleles from *C. australis* (CtauDRB01 and CtauDQA01) are reported in Cutrera & Lacey (2007). The remaining DRB and DQA alleles detected for this species are reported here (DRB exon 2: GQ497462–GQ497470; DQA exon 2: GQ497448–GQ497454; DQA intron 2: GQ497455–GQ497461). The MHC alleles analysed for *C. talarum* are reported in Cutrera *et al.* (2006). Comparisons of the study species revealed that the number of MHC alleles detected was greater for *C. australis* at both exons and the intron examined (Table 2). By contrast, observed heterozygosity for all three MHC regions was greater for *C. talarum* (Table 3). Comparisons of allele frequency distributions revealed a highly skewed distribution in *C. australis* (Fig. 2). Thus, although more polymorphic sites were detected for *C. australis*, haplotype diversity, the number of pairwise differences between alleles, and nucleotide diversity were lower for this species for all MHC regions considered (Table 2). In both species, the DRB and DQA exons exhibited higher levels of molecular polymorphism and heterozygosity than the DQA intron (Tables 2, 3), with r/θ ratios of approximately 1 for each exon (Table 4). The estimated minimum number of recombination events was 0 for most of the regions analysed; one possible recombination event was detected for DQA exon 2 in *C. australis* (Table 4). Significant evidence of recombination was detected only for DQA intron 2 from *C. talarum* (Table 4). These findings suggest that mutation and recombination have had little impact on variability at the MHC exons examined in the present study.

Departures from neutrality based on MHC polymorphisms

Hardy–Weinberg (H–W) and Ewens–Watterson (E–W) tests revealed no evidence of selection on the DQA intron for either study species (H–W exact test, $P = 1$, Table 3; E–W: $F_{C.\ talarum} = 0.62$, $F_{C.\ australis} = 0.81$; Watterson's F, $P = 1$; Slatkin's exact test, $P = 1$; Slatkin, 1994); these results are consistent with the expectation that this intron represents a selectively neutral, noncoding portion of the MHC complex. For *C. talarum*, both tests revealed significant departures from neutrality for the DQA exon (H–W exact test, $P < 0.0001$, Table 3; E–W: $F = 0.36$; Watterson's F, $P = 0.029$; Slatkin's exact test, $P = 0.031$; Slatkin, 1994; Cutrera & Lacey, 2006) but no evidence of selection on the DRB exon (H–W exact test, $P = 1$, Table 3; E–W: $F = 0.41$; Watterson's F, $P = 1$; Slatkin's exact test, $P = 1$; Slatkin, 1994; Cutrera & Lacey, 2006). For *C. australis*, no evidence of selection was found for either exon (H–W exact test, $P_{DRB,\ DQA} = 1$, Table 3; E–W: $F_{DRB} = 0.76$, Watterson's F, $P_{DRB} = 1$; Slatkin's exact test, $P_{DRB} = 1$, Table 3; $F_{DQA} = 0.58$; Watterson's F; $P_{DQA} = 0.97$; Slatkin's exact test, $P_{DRB} = 0.97$).

For *C. talarum*, values of Tajima's D and Fu's F_S were positive for both exons but negative (D) or nearly zero (F_S) for the DQA intron (Cutrera & Lacey, 2006); this outcome was significant for D for the DQA exon and F_S for the DRB exon (Table 3). By contrast, for *C. australis*, estimates of Tajima's D and Fu's F_S were significantly negative for all three MHC regions examined (Table 3). For the mitochondrial control region, values of D and F_S for each species were generally consistent with those obtained for the DQA intron, although the negative values obtained for the control region from *C. australis* were not significantly different from zero (Table 3). Collectively, these findings suggest that different processes or events have influenced MHC variation in the study species.

Table 2. Major histocompatibility complex (MHC) diversity in 34 *Ctenomys australis* and 30 *Ctenomys talarum* from Necochea, Argentina

Species	Locus	Number of alleles/haplotypes	S	H_D	π	k	Source
Ctenomys australis	DRB exon2	10	27	0.249 (0.07)	0.003 (0.002)	0.823 (0.282)	Present study
	DQA exon2	9	12	0.424 (0.07)	0.004 (0.003)	0.723 (0.248)	Present study
	DQA intron2	8	10	0.197 (0.065)	0.001 (0.005)	0.352 (0.121)	Present study
	mtDNA control region	7	7	0.820 (0.119)	0.006 (0.003)	2.436 (0.599)	Mora *et al.* (2006); Present study
Ctenomys talarum	DRB exon2	6	8	0.608 (0.034)	0.008 (0.0004)	3.53 (0.906)	Cutrera & Lacey (2006)
	DQA exon2	3	3	0.64 (0.29)	0.0082 (0.004)	1.45 (0.5)	Cutrera & Lacey (2006)
	DQA intron2	4	4	0.406 (0.101)	0.01 (0.001)	0.7 (0.285)	Cutrera & Lacey (2006)
	mtDNA control region	6	7	0.7 (0.114)	0.004 (0.002)	1.619 (1.013)	Mora *et al.* (2007); present study

The number of alleles or haplotypes, number of segregating sites (S), haplotype diversity (H_D), nucleotide diversity per locus (π), and mean number of pairwise nucleotide differences between all sequences sampled (k), are shown for DRB exon 2, DQA exon 2, and DQA intron 2. For k, standard deviations are given in parentheses. mtDNA, mitochondrial DNA.

Table 3. Results of tests to detect departures from neutrality for major histocompatibility complex (MHC) loci and the mitochondrial DNA (mtDNA) control in *Ctenomys australis* and *Ctenomys talarum*

		Test			
				Hardy–Weinberg	
Species	Locus	Tajima's *D*	Fu's *F*	H_O	H_E
Ctenomys australis	DRB exon 2	–2.696* (–1.525,1.871)	–5.87* (–3.449,4.274)	0.265	0.274
	DQA exon 2	–2.000* (–1.429,1.909)	–3.94* (–3.619,4.719)	0.441	0.446
	DQA intron 2	–2.255* (–1.667,1.560)	–7.64* (–3.528,4.223)	0.206	0.223
	mtDNA control region	0.08	–2.26	–	–
Ctenomys talarum	DRB exon 2	0.615 (–1.574,1.664)	3.61* (–3.389,3.337)	0.63	0.6
	DQA exon 2	2.480 * (–1.624,2.059)	1.59 (–3.636,4.762)	0.649	0.583*
	DQA intron 2	–0.117 (–1.640,1.338)	0.46 (–3.475,4.129)	0.393	0.389
	mtDNA control region	–0.89	–1.4	–	–

For MHC loci, lower and upper limits of 95% confidence intervals for Tajima's *D* and Fu's *F* (expected under neutrality and considering recombination) estimated using DNAsp, version 4 (Rozas *et al.*, 2003) are given in parentheses. H_O, observed heterozygosity; H_E, expected heterozygosity. *Denotes significant departures from Hardy–Weinberg expectations ($P < 0.05$) and also indicate those values of Tajima's *D* or Fu's *F* that were not contained within the associated 95% confidence intervals.

Evidence of selection based on nucleotide substitution rates

Omega (ω) values for both MHC exons were larger for *C. australis*. For DRB, likelihood ratio tests revealed no significant differences in fit between any of the seven nucleotide substitution models considered (Table 5). Similarly, no significant differences in fit were found for the DQA exon from *C. talarum*. By contrast, for DQA from *C. australis*, the comparisons between models M1a and M2a, M7 and M8, and M8a and M8 were significant (Table 5), suggesting that, for this species, selection is acting on specific codons within this exon. Specifically, residues 11 and 14 of the DQA exon from *C. australis* were identified as being subject to positive selection. Thus, comparisons of nucleotide substitution patterns are consistent with analyses of MHC polymorphism in providing greater evidence of selection at the DQA locus. Details of the function parameters are provided in Table S1 (Appendix).

Inferring demographic history from microsatellite variation

Maximum likelihood analyses conducted in LAMARC suggested a difference in demographic history between the two species. Under both the stepwise and Brownian mutation models, microsatellite variation in *C. australis* produced positive estimates of *g* that were consistent with a demographic expansion ($g_{STEPWISE}$ = 7.209; 99% CI = 3.897–10.617; $g_{BROWNIAN}$: 4.751; 99% CI = 3.027–7.589). CIs for these estimates did not include 0, suggesting significant departures from the null expectation of no historical change in population size. By contrast, estimates of *g* obtained for *C. talarum* were much closer to zero ($g_{STEPWISE}$ = 0.493; 99% CI = 0.262–0.782; $g_{BROWNIAN}$: 0.187; 99% CI = 0.087–0.432). Although confidence intervals for these estimates also excluded 0, the markedly smaller values of *g* for this species suggest that population size has historically been more stable in *C. talarum*. By contrast, analyses using BOTTLENECK revealed no differences between observed microsatellite allele frequencies and the L-shaped distribution characteristic of populations that have not experienced a recent bottleneck. For both study species, the number of loci for which $H_E > H_{EQ}$ did not differ significantly from that expected under equilibrium conditions (*C. australis*: Wilcoxon signed rank test, $P = 0.820$; *C. talarum*: Wilcoxon signed rank test, $P = 0.074$, Cutrera *et al.*, 2006), providing no evidence of historical reductions in population size in either of the study species.

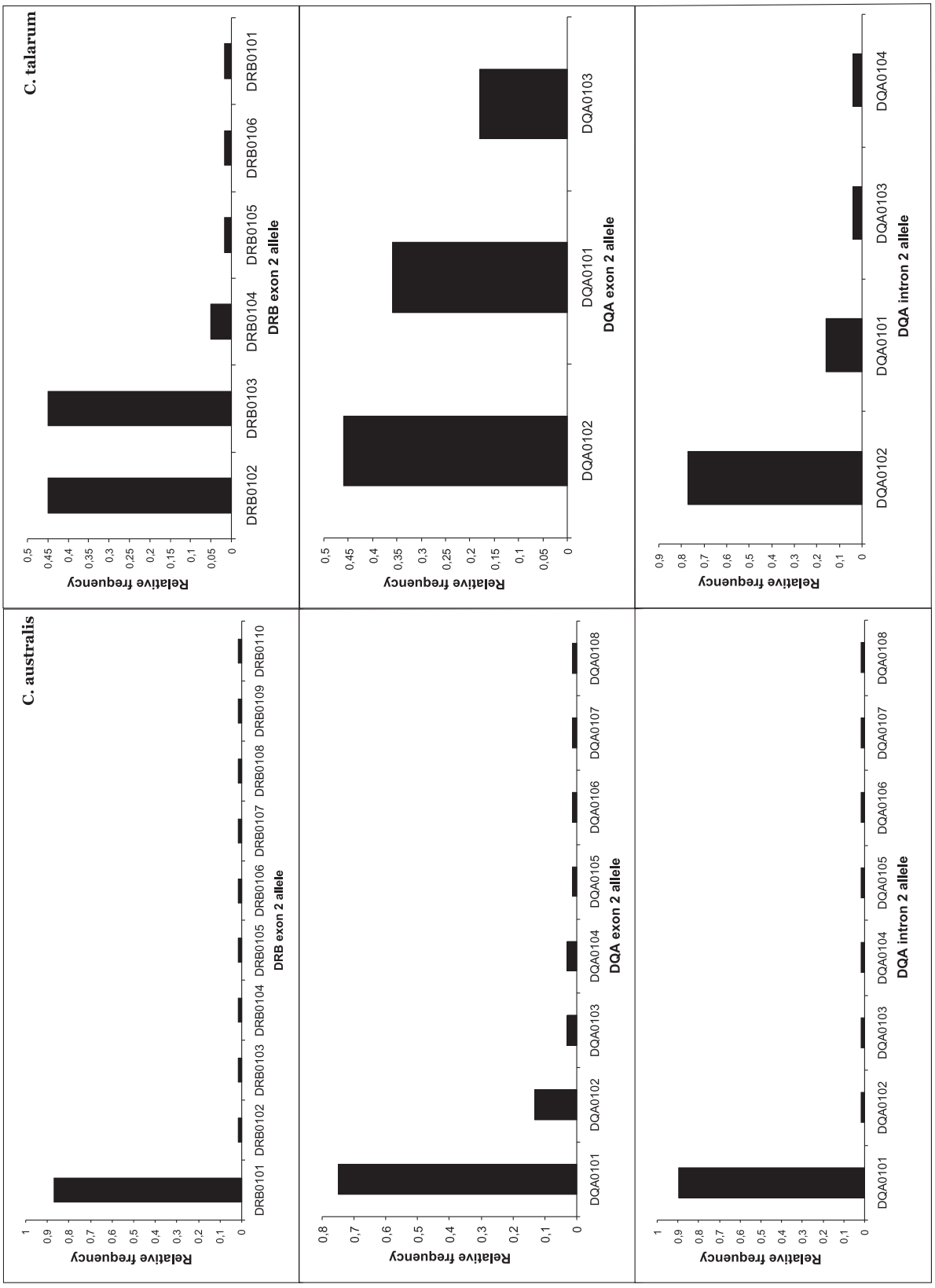

Figure 2. Histograms of allele frequencies for major histocompatibility complex (MHC) loci from *Ctenomys australis* (left panels) and *Ctenomys talarum* (right panels). Data are from DRB exon 2, DQA exon 2, and DQA intron 2.

Table 4. Analyses of recombination at major histocompatibility complex (MHC) class II DRB and DQA loci in *Ctenomys australis* and *Ctenomys talarum*

Locus and species	*N*	θ	*r*	*r*/θ	*Rm*
DRB exon 2					
Ctenomys australis	68	5.638	2.02	0.358	0
Ctenomys talarum	60	1.703	6.061	3.559	0
DQA exon 2					
Ctenomys australis	68	1.51	2.506	1.66	1
Ctenomys talarum	60	0.643	0	0	0
DQA intron 2					
Ctenomys australis	68	2.088	2.021	0.968	0
Ctenomys talarum	60	0.871	3.518*	4.039	0

For each locus, maximum likelihood estimates of the population recombination parameter (*r*) and the finite sites population mutation parameter (θ) are shown; these values were estimated using LDhat (McVean *et al.*, 2002). The minimum number of recombination events (*Rm*), estimated using DNAsp, version 4 (Rozas *et al.*, 2003), is also shown. N, number of sequences used. *Statistically significant value of *r* ($P < 0.001$), as determined by the permutation approach implemented in LDhat (McVean *et al.*, 2002).

Table 5. Maximum likelihood analyses of models of codon evolution for the major histocompatibility complex (MHC) class II DRB and DQA loci in *Ctenomys australis* and *Ctenomys talarum*

Locus					
		DRB exon 2		DQA exon 2	
Species	Models compared	2ΔlogL	*P* (χ^2)	2ΔlogL	*P* (χ^2)
Ctenomys australis	M1a – M2a	0.92	0.63	8.86*	0.012
	M0 – M3	0	1	7.58	0.108
	M7 – M8	0.9	0.64	9.01*	0.01
	M8a – M8	0.9	0.34	8.85*	0.002
Ctenomys talarum	M1a – M2a	0.26	0.878	0	1
	M0 – M3	0	1	0.26	0.99
	M7 – M8	0.26	0.88	0	1
	M8a – M8	0.26	0.61	0	1

Analyses were completed using CODEML (included in PAML, version 3.14). *Significant differences in fit ($P < 0.05$) between models M1a and M2a, M7 and M8, and M8a and M8 (explanations of these models are provided in the text).

Comparisons of MSTs

MSTs for the DRB and DQA exons indicated that MHC allele frequency distributions tended to be more biased toward a single, common allele in *C. australis* (Fig. 3). By comparison, allele frequencies for *C. talarum* were more evenly distributed among a smaller number of variants (Figs 2, 3). The topologies of the MSTs for these exons also clearly differed between species. MSTs for *C. australis* had a generally 'star-like' appearance characterized by a single, high-frequency allele surrounded by a number of closely related, low-frequency alleles and one or two more distantly related alleles. By contrast, MSTs for *C. talarum* were more linear in structure, with less variation in the number of substitutions separating adjacent alleles. MSTs for both the DQA intron and mitochondrial control region revealed similar interspecific differences in topology (Fig. 3), suggesting that processes and events underlying these differences have impacted neutral as well as functional regions of the genome.

DISCUSSION

The findings obtained in the present study support the assertion that demographic history has contributed substantially to differences in patterns and levels of MHC variability in the two sympatric species

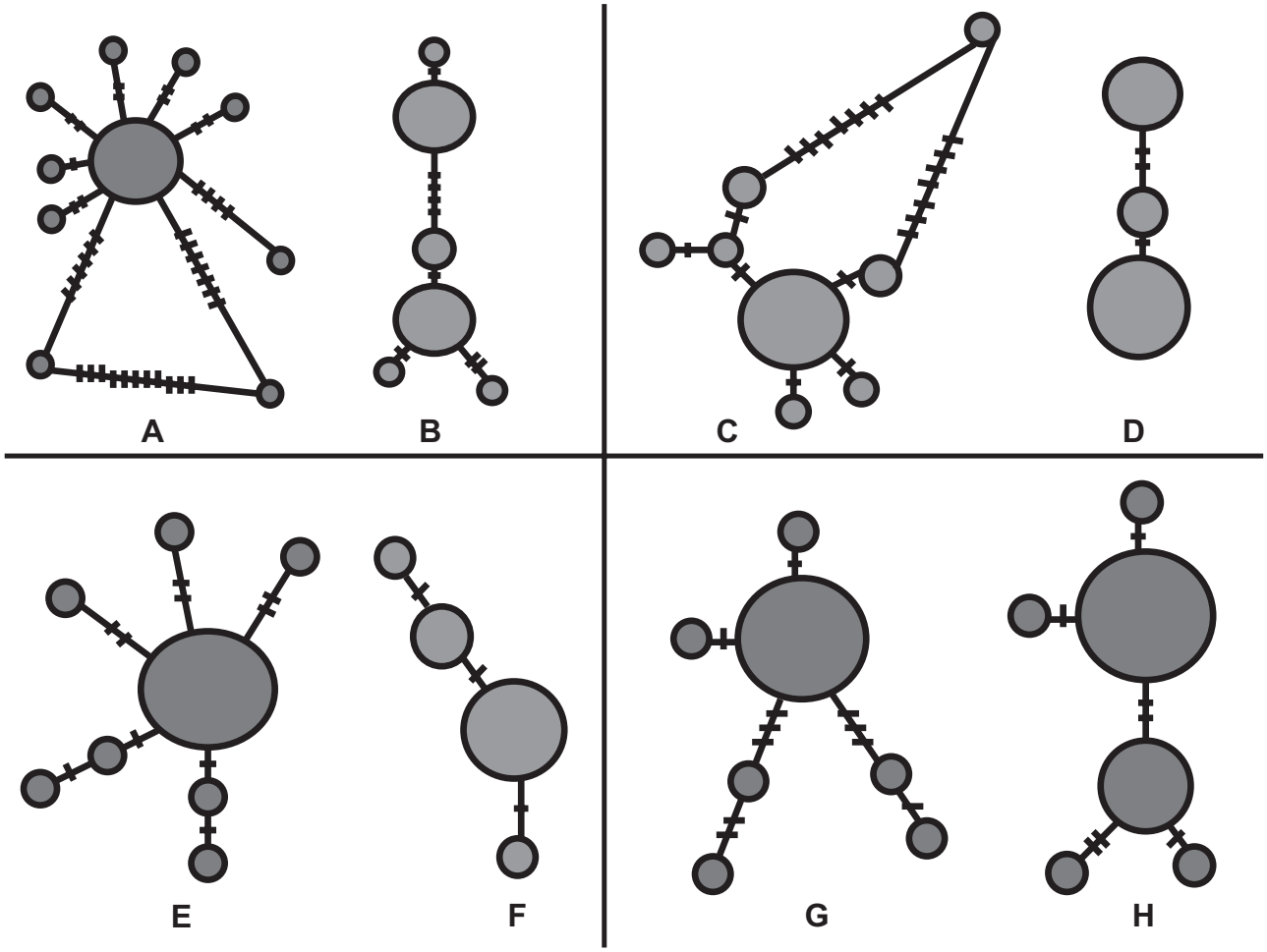

Figure 3. Minimum spanning trees (MSTs) for major histocompatibility complex (MHC) loci from *Ctenomys australis* (left panels) and *Ctenomys talarum* (right panels). Data are from DRB exon 2 (A, B), DQA exon 2 (C, D), DQA intron 2 (E, F), and the mitochondrial DNA control region (G, H). Circle sizes are proportional to the frequency of each MHC allele or mitochondrial DNA haplotype. Control region MSTs are modified from Mora *et al.* (2006).

Table 6. Summary of evidence for selection on the major histocompatibility complex (MHC) class II loci DRB and DQA loci in *Ctenomys australis* and *Ctenomys talarum*

Species	Locus	H–W	E–W	Tajima's *D*	Fu's *F*	ω	Demographic history
Ctenomys australis	DRB exon 2	NS	NS	–2.696*	–5.87*	NS	Recent population expansion
	DQA exon 2	NS	NS	–2.000*	–3.94*	*	
Ctenomys talarum	DRB exon 2	NS	NS	0.615	3.61*	NS	Stability
	DQA exon 2	*	*	2.480*	1.59	NS	

The results of Hardy–Weinberg (H–W) and Ewens–Watterson (E–W) are shown, as are values for Tajima's *D*, Fu's F_S, and ω (the selection parameter, equivalent to d_N/d_S; Goldman & Yang, 1994). A brief description of the apparent demographic history of each study population is also included, as determined from analyses of neutral markers. NS, departures from values expected under neutrality are not significant. *Significant results ($P < 0.05$).

of tuco-tucos that were the focus of the study (Table 6). Previous analyses of mtDNA sequences from these animals suggested that, although *C. talarum* has experienced a stable demographic history consistent with populations at mutation–drift equilibrium (Mora *et al.*, 2007), *C. australis* appears to have undergone a recent demographic expansion (Mora *et al.*, 2006). On the basis of this apparent

historical difference, we predicted that allele frequency distributions should be more skewed, differences between coding and noncoding regions should be less pronounced, and evidence of current selection should be reduced in *C. australis* relative to *C. talarum*. The first two of these predictions appear to be met by our data. By contrast to our expectation, however, greater evidence of positive selection on MHC exons was detected for *C. australis*.

Inferences regarding demographic history

The apparent historical differences between the study species led Mora *et al.* (2006) to propose that Quaternary changes in sea level increased the availability of the sand dune habitat in which *C. australis* occurs, thereby prompting a rapid expansion of populations of this species. Paleontological data support this scenario; the oldest fossilized remains for this species are dated at approximately 9000 ± 50 years BP (Pardiñas, 2001), suggesting that the appearance of *C. australis* in the coastal dunes of eastern Argentina may have been associated with the expanding availability of this habitat type. The analyses of data from Necochea in the present study are consistent with this scenario. Specifically, the topologies of control region minimum spanning trees, the results of Tajima's D and Fu's F_S tests, and estimates of the population growth parameter g suggest a moderate demographic expansion in *C. australis* but not *C. talarum* (Tajima, 1989; Kuhner, 2006); no evidence of historical reductions in population size (e.g. bottlenecks) were found for either species. Although other processes (e.g. selective sweeps) can produce a pattern of genetic structure similar to that observed in *C. australis*, the strong concordance across markers reported in the present study suggests that the differences in neutral genetic structure between the study populations reflect historical demographic events, specifically a demographic expansion for *C. australis*.

Impacts of history on MHC variation

Multiple lines of evidence suggest that the distinct demographic histories of the study populations have influenced MHC variation in these animals. For example, comparisons of allele frequency distributions, measures of nucleotide diversity, and MST topologies for the MHC exons examined are consistent with the apparent interspecific differences in demographic history revealed by neutral markers; in particular, the scenario of a recent expansion in *C. australis* (Slatkin & Hudson, 1991; Harpending *et al.*, 1998). With regard to variation at functional versus neutral portions of MHC genes, estimates of Tajima's D and Fu's F_S suggest greater concordance between these regions in *C. australis*, with the excess of low frequency alleles (significantly negative values of D and F_S) expected subsequent to a recent expansion (Tajima, 1989). Thus, the observed patterns of exonic variation support the first two of our predictions regarding the effects of demographic history on MHC loci and corroborate our analyses of neutral markers in suggesting that *C. australis* has experienced a recent demographic expansion.

Possible alternative explanations for the observed differences in MHC variability between the study populations include selection against slightly deleterious mutations at closely linked exons or a selective sweep; either of these processes acting on *C. australis* could have contributed to the pattern of allelic diversity detected for these animals (Simonsen, Churchill & Aquadro, 1995; Nielsen, 2005). Because the DQA intron is closely linked to the polymorphic exon 2 (Brown *et al.*, 1993), positive selection on this exon may have contributed to reduced variation at the intron (i.e. genetic hitchhiking: Kaplan, Hudson & Langley, 1989; Nielsen *et al.*, 2005; but see also Cereb, Hughes & Yang, 1997), leading to similar patterns of variability for these regions. This outcome was not observed in *C. talarum*, indicating that close physical linkage does not consistently result in similar patterns of variability at adjacent MHC regions. Concomitantly, for *C. australis*, analyses of the mitochondrial control region (presumably a neutral marker not linked to the DQA locus) yielded results that generally were similar to those for the DQA intron, suggesting that the processes affecting intronic variation in this population are not limited to hitchhiking with closely-linked exons (Stephan, Wiehe & Lenz, 1992; Jensen *et al.*, 2005).

Because class II MHC molecules such as DRB and DQA bind foreign peptides and mediate the associated activation of T-cells (Apanius *et al.*, 1997), specific MHC alleles may be associated with response to particular pathogens and selective sweeps in response to pathogen outbreaks have been invoked to explain apparent reductions in MHC allelic diversity in some species (de Groot *et al.* 2002). Such events should affect only the functional portions of the genome (Nielsen, 2005); given that significantly negative values of D and F_S were obtained for the intron as well as the exons examined for *C. australis*, it is unlikely that a selective sweep alone can account for the differences in MHC variability between the study species. Distinguishing between the effects of selection and demographic history is difficult (Nielsen, 2005) and remains a significant challenge to studies investigating the processes underlying variation at functional loci. With regard to our data, the apparent inability of alternative (i.e. selective) scenarios to

account completely for the observed patterns of variability, combined with the multiple lines of evidence indicating a recent demographic expansion in *C. australis*, suggests that differences in demographic history have played a significant role in generating the distinct patterns of MHC variability that are evident in the study populations.

Interspecific differences in selection on MHC genes

Given that a rapid demographic expansion is likely to have enhanced the role of genetic drift in shaping genetic variation in *C. australis* (Mora *et al.*, 2006), we had expected that evidence of recent selection on MHC genes should be less pronounced in this species than it is in *C. talarum*. Data regarding departures from neutrality were consistent with this prediction; only data from *C. talarum* revealed significant departures from neutral expectations for the DRB and DQA loci. By apparent contrast, however, values of ω suggested that positive selection on DQA has been stronger in *C. australis*. Further consideration of these data revealed that the study species differ with respect to the apparent time frame over which selection appears to have acted. For *C. talarum*, the results obtained with respect to H–W, E–W, and Tajima's *D* are consistent with recent or ongoing balancing selection on the DQA exon (Harris & Meyer, 2006), whereas Fu's F_S test supports a similar scenario for DRB. By contrast, evidence of selection in *C. australis* is based on patterns of codon variation that are less sensitive to demographic change (Garrigan & Hedrick, 2003; Harris & Meyer, 2006), suggesting that positive selection has contributed to MHC variation in this species over a longer evolutionary time frame, as required for the accumulation of multiple mutations.

These findings suggest that selection is a dynamic process, the effects of which may vary temporally. Because the relevant demographic differences between the study populations are considered to reflect Quaternary changes in habitat availability (Isla, 1998; Mora *et al.*, 2006), their effects should be most evident in terms of recent selection on MHC genes, an expectation that is consistent with the observed outcomes of H–W and E–W tests for *C. talarum* and *C. australis*. By comparison, changes in selection over longer time frames may reflect cumulative temporal variation in the composition or distribution of pathogens (Hedrick, Lee & Garrigan, 2002), which are considered to be the primary selective agents operating on MHC genes (Bernatchez & Landry, 2003; Piertney & Oliver, 2006). The finding in the present study demonstrating that the timeline over which selection appears to have acted differs between the study populations adds to a growing body of evidence indicating that temporal fluctuations may be as important as spatial heterogeneities in shaping the genetic responses of wild populations to selection (Westerdahl *et al.*, 2004; Charbonnel & Pemberton, 2005; Jensen *et al.*, 2008; Oliver *et al.*, 2009).

Data indicating that individual class II alleles may be associated with specific pathogens (Paterson, Wilson & Pemberton, 1998; Froeschke & Sommer, 2005) also imply variation in how MHC loci respond to environmental or demographic conditions. In both of our study populations, the DRB exon displayed greater allelic variability than the DQA exon, and this pattern has been reported for multiple other species (Gyllensten *et al.*, 1994; Kennedy *et al.*, 2000; Seddon & Ellegren, 2002; Weber *et al.*, 2004). The intensity of selection also tends to be greater for DRB (Satta *et al.*, 1994), suggesting a consistent, perhaps functional basis for differences in variability between these loci. Both of the exons examined in the present study are considered to be involved in binding foreign peptides. Although the distributions of nonsynonymous substitutions detected were consistent with immunologically active MHC molecules, the specific functions of these genes remain poorly characterized. Thus, it is possible that locus-specific differences in activity or the nature of the peptides detected may have contributed to the observed differences in variability and selection at these genes.

Implications for MHC evolution

Previous analyses of MHC variation in tuco-tucos (Cutrera & Lacey, 2007) have revealed evidence of positive selection acting over the evolutionary history of the genus *Ctenomys*, resulting in the maintenance of allelic lineages that predate species divergence events. The results obtained in the present study suggest that allele frequency distributions at MHC loci in *C. australis* have been shaped largely by recent demographic history, although the signature of positive selection acting over longer time periods is still evident at these genes. Most studies of historical effects on MHC loci have focused on species known to have undergone severe reductions in population size (Mikko & Andersson, 1995; Seddon & Baverstock, 1999; Seddon & Ellegren, 2002). In these taxa, history appears to have acted primarily to reduce MHC diversity, with variable evidence of subsequent recovery and selection on MHC loci. As noted by Hedrick *et al.* (2000) and Hedrick, Lee & Garrigan (2002), a bottleneck followed by a population expansion tends to produce alleles that are more divergent from each other than would be expected by chance, suggesting that selection favours the retention of functionally distinct variants. Although a similar pattern is

evident for *C. australis*, analyses of neutral molecular markers provide no evidence of a recent reduction in population size at Necochea (Cutrera *et al.*, 2006; present study). Instead, as argued above, the primary historical event affecting genetic diversity in these animals appears to have been a rapid demographic expansion. This conclusion emphasizes the importance of historical factors in shaping MHC variation at the same time as generating new insights into the interactions between demography and selection that produce complex signatures of selection on MHC loci. Studies of other species of tuco-tucos, including other species pairs that occur in close physical proximity but have been subject to contrasting demographic processes, should add to our understanding of how demography and selection interact to shape MHC evolution over larger geographic distances and deeper time scales.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Results of maximum likelihood analyses of models of codon evolution for the major histocompatibility complex (MHC) Class II DRB and DQA loci (exon 2) in *Ctenomys australis* and *Ctenomys talarum*. Analyses were completed using CODEML (included in PAML, version 3.14). A, results for DRB exon 2. B, results for DQA exon 2. Significant differences in fit between models M1a and M2a, M7 and M8, and M8a and M8 are denoted in bold. ω is the selection parameter (equivalent to d_N/d_S; Goldman & Yang, 1994) and p_n is the proportion of sites that fall into the ω_n site class. For models M7, M8a and M8, p is the shape parameter of the β function.

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