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Creating new evolutionary pathways through bioinvasion: the population genetics of brushtail possums in New Zealand

STEPHEN D. SARRE,* NICOLA AITKEN,*[1] AARON T. ADAMACK,* ANNA J. MACDONALD,* BERND GRUBER* and PHIL COWAN†

Wildlife Genetics Laboratory, Institute for Applied Ecology, University of Canberra, Bruce, ACT 2601, Australia, †Landcare Research New Zealand, Lincoln 7640, New Zealand

Abstract

Rapid increases in global trade and human movement have created novel mixtures of organisms bringing with them the potential to rapidly accelerate the evolution of new forms. The common brushtail possum (*Trichosurus vulpecula*), introduced into New Zealand from Australia in the 19th century, is one such species having been sourced from multiple populations in its native range. Here, we combine microsatellite DNA- and GIS-based spatial data to show that *T. vulpecula* originating from at least two different Australian locations exhibit a population structure that is commensurate with their introduction history and which cannot be explained by landscape features alone. Most importantly, we identify a hybrid zone between the two subspecies which appears to function as a barrier to dispersal. When combined with previous genetic, morphological and captive studies, our data suggest that assortative mating between the two subspecies may operate at a behavioural or species recognition level rather than through fertilization, genetic incompatibility or developmental inhibition. Nevertheless, hybridization between the two subspecies of possum clearly occurs, creating the opportunity for novel genetic combinations that would not occur in their natural ranges and which is especially likely given that multiple contact zones occur in New Zealand. This discovery has implications for wildlife management in New Zealand because multiple contact zones are likely to influence the dispersal patterns of possums and because differential susceptibility to baiting with sodium fluoroacetate between possums of different origins may promote novel genetic forms.

Keywords: barriers to dispersal, hybrid zones, invasive species, microsatellite DNA, sympatry

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Introduction

Rapid increases in global trade and human movement have created novel combinations of organisms and brought into sympatry many species and varieties that were previously allopatric. In many cases, these introduced species can become invasive in their new environments and have profound impacts on native species (e.g. Sacks *et al.* 2010). As a result, the increased rate at which species are invading new environments is a principle driver of biological extinctions and ecosystem change (Wilcove *et al.* 1998). While the introduction of small numbers of individuals from a single species might lead to problems of inbreeding and low genetic variation in founding populations (Le Roux & Wieczorek 2009), the sourcing of introductory populations from multiple sites may actually increase genetic variation by admixing previously separated gene pools, thereby increasing the potential for evolutionary change (Lee 2002). Admixture may also enhance the creation of novel forms through recombination between different

Correspondence: Stephen D. Sarre, Fax: +61 (2) 6201 5305; E-mail: stephen.sarre@canberra.edu.au
[1]Current address: Australian National University, Canberra, ACT 0200, Australia

genotypes and by masking deleterious mutations that arise through local adaptation in isolated source populations (Verhoeven *et al.* 2011). Thus, the combined effects of new environments, reduced exposure to coevolved pathogenic organisms and novel genetic combinations provide opportunities for the rapid evolution of new forms of invading species.

New Zealand, like many countries colonized by Europeans in the 19th century, was subject to active introductions of terrestrial mammals, creating an admixture of mammalian fauna that is unique (Clout 2001). Of these introduced mammals, the common brushtail possum (*Trichosurus vulpecula*, Kerr, 1792), an Australian marsupial introduced for its fur (Pracy 1974), is widely considered that country's most important pest through its role in forest destruction, predation on native species and the transmission of bovine tuberculosis (Parkes & Murphy 2003). Brushtail possums have a well documented but complex history of introduction to New Zealand (Pracy 1974). They were obtained from Tasmania and several localities on the Australian mainland (Victoria and possibly New South Wales) and imported into New Zealand on 36 separate occasions between 1837 and 1924 (Thomson 1922; Pracy 1974). These importations were followed by over 250 separate liberations conducted among localities. Records of the sources and destinations of these liberations are incomplete, but the introduction of at least two forms of possums has been well recognized in New Zealand, primarily because of the distinctive coat colour differences between the animals of Tasmanian origin (predominantly black or reddish black coat) compared with those from the Australian mainland (predominantly grey).

Although high levels of morphological variability are evident, common brushtail possums are considered a single species in Australia (Kerle *et al.* 1991) albeit a species within which five geographically based subspecies including *Trichosurus v. fuliginosis* from Tasmania and *T. v. vulpecula* from southern Australia are recognized (How & Kerle 1995). In New Zealand, there is a wide acceptance of the single species status with almost exclusive reference to a single taxon *Trichosurus vulpecula* in key reviews (e.g. Clout & Sarre 1997; O'Reilly-Wapstra & Cowan 2010) even though differences between the two forms are evident. These differences include susceptibility to the widely used poison sodium fluoroacetate (McIlroy 1983) with significant differences observed between possums that were grey, most likely *T. v. vulpecula* (LD_{95} = 2.9 mg/kg) and dark, most likely *T. v. fuliginosus* (LD_{95} = 4.1 mg/kg; Henderson *et al.* 1999).

The localized distribution of possums of different origins will be affected by the rate at which they disperse and the nature of the interactions between the different subspecies as well as their introduction history. It is well established that possum dispersal is male biased (Clout & Efford 1984; Ji *et al.* 2001) and is also likely to be influenced by significant landscape features such as large rivers (Cowan & Clout 2000) and localized depopulation through control operations (Cowan *et al.* 1997; Ji *et al.* 2004). However, we can find no published examination of the nature of the interaction between the two subspecies, *T. v. fuliginosis* and *T. v. vulpecula*, leaving open the possibility that dispersal by one subspecies may be influenced by the presence of the other through behavioural or other interactions.

Here, we analyse the spatial population genetic distribution of microsatellite DNA genotypes of possums in Hawke's Bay, New Zealand. *T. v. fuliginosis* were released at several sites in this region from 1890 to the 1920s followed by the release of *T. v. vulpecula* at two sites to the south of the study area in 1936. Our hypothesis was that the two subspecies released in the area would form a single panmictic population of novel genotypes not possible in Australia because of allopatry. We also predicted that the presence of major rivers in the area would represent the most significant barrier to dispersal in this region where a barrier may differ in its level of porosity. Our analysis, using a combination of STRUCTURE, spatial principal component and least-cost path approaches, reveals a key role for rivers in inhibiting dispersal but also identifies a clear genetic distinction among possum populations of different Australian origin and identifies a zone of introgression between the two.

Materials and methods

Study area

The study area was located in the north-western Hawke's Bay area of New Zealand (Fig. 1). The introduction of possums into the area began in 1898 at Lake Waikaremoana with the release of 10 possums imported directly from Tasmania (Fig. 1; Pracy 1974), and later with black coated animals, also of Tasmanian origin, translocated from elsewhere in New Zealand to Lake Waikaremoana and the Mahia Peninsula. Grey-coated animals, most likely originating from the Australian mainland, were translocated to the Mohaka River and locations further south. Documented translocations into the area ceased in 1936. In this study, possums were sampled along six transects from 31 sites that were separated by up to 61 km, a distance that exceeds the range of possum dispersal distances observed in previous studies (Cowan & Clout 2000) and included sites that were paired across major rivers (Fig. 1). A GPS reading, sex and coat colour were recorded at the point of each cap-

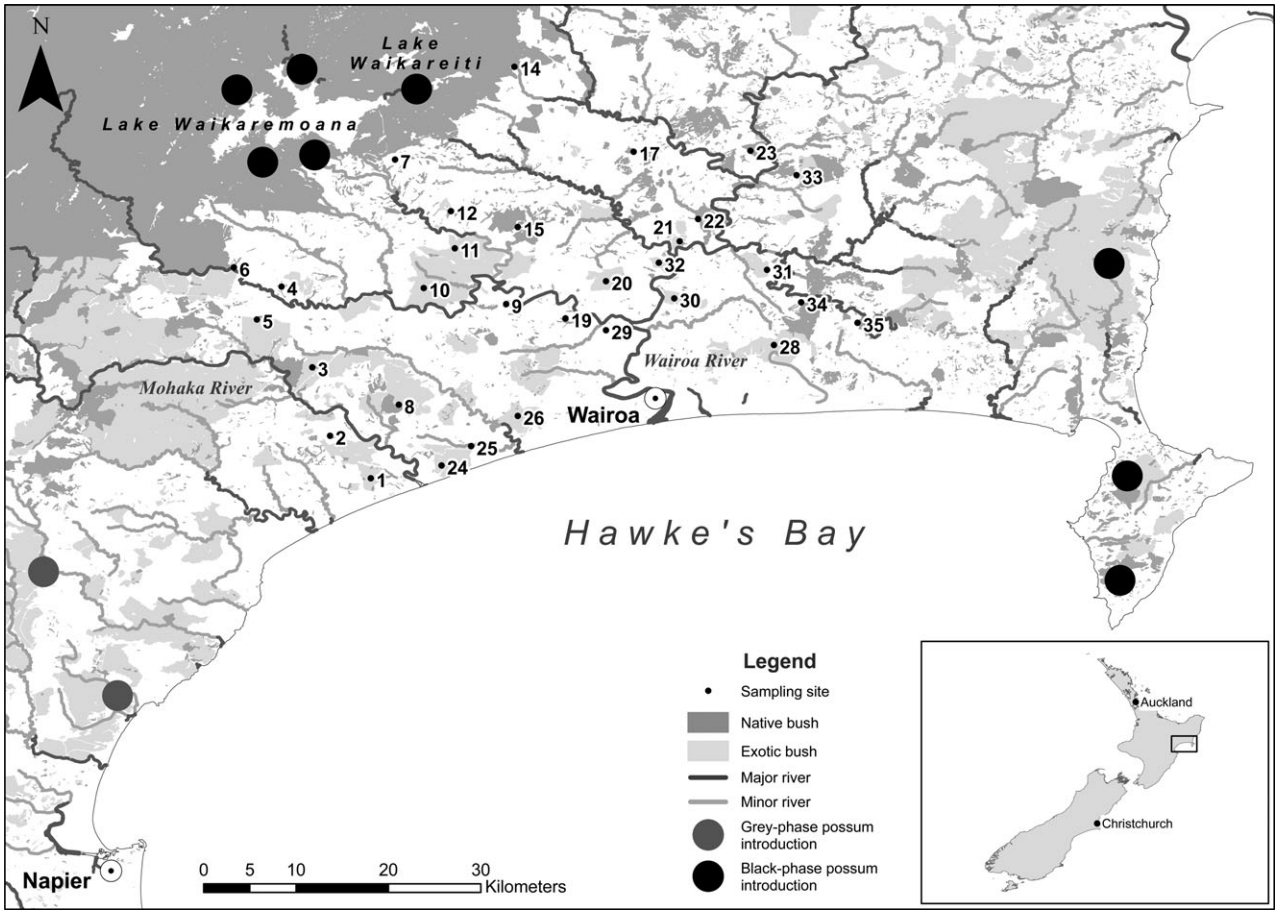

Fig. 1 Possum sampling sites and the approximate sites of possum introductions in the north-west Hawke's Bay region of New Zealand.

ture for each animal, and an ear tissue sample was collected and stored long-term in individual 2-mL tubes in 80% ethanol at −80 °C. At the time of sampling (2004), formal animal ethics approval for killing of wild animal pests, such as possums for tissue collection, was not required under New Zealand welfare legislation. Possums were either live trapped, stunned and exsanguinated or were poisoned with cyanide baits used for possum control, both common practices used during possum harvesting for fur at the time. All animals were sampled within a 3-month period from July to September.

DNA analysis

DNA was extracted from 1790 possum ear tissue samples as follows. Approximately, 20 mg of ear tissue was incubated overnight at 55 °C in 150 μL of digestion solution containing 10%w/v Chelex®-100 (BioRad) and 0.27 μg/μL proteinase K (Amresco) in distilled water. The resultant supernatant was removed, and the solution was incubated at 99 °C for 10 min and then diluted 1 in 40 in 0.2 M trehalose (Sigma T5251). About 1.5 μL aliquots were dried and stored at room temperature.

Possums were genotyped for variation at nine microsatellite DNA loci. Five of these were developed by Taylor & Cooper (1998; Tv16, Tv19, Tv53, Tv58, Tv64), one locus (TvM1) was reported by Lam *et al.* (2000), one locus (Tv5.64) was developed from an enriched library created for the study reported here, and two loci (B38.1 and Tv_PnMs16), were modified for possums following cloning and sequencing from loci originally isolated from other marsupials by Zenger & Johnston (2001) and Millis (2000) respectively (Table 1). PCRs contained 1 × PCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8), 0.1% Tween-20), 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.3 μM each primer (except 0.2 μM for Tv53), 0.3 units *Taq* polymerase (Bioline), H_2O to a final volume of 10 μL with the forward primer fluorescently labelled for subsequent genotyping. Multiplex reactions containing Tv19 and Tv58, TvPnMs16 and TvB38.1, Tv16 and Tv53 were performed. All other loci

Table 1 Locus details, PCR annealing temperature and source for the unpublished loci used in this study

Locus Name	Microsatellite sequence	Primer (forward)	Primer (reverse)	Annealing temp. (°C)	Source	GenBank accession no.
Tv5.64	$(GATA)_{14}$	TTTATCCCTACTAgAggTAggT	CCCTCTCCATCTgCTCCTC	52	This study	HM448904
Tv6.9	$(GATA)_2GAT(GATA)_{17}$	GGAgCCAACTgTgCgACTg	AACgCCTTCACgTATgAACTC	55	This study	HM448905
Tv6.22	$GATA(GA)_2(GATA)_7[GAT(GATA)_3]_2(GATA)_{13}$	CCATgATTAggATgggAAAC	ggCCCgTgACACACAAATATC	50	This study	HM448906
Tv_PnMs16	$(CA)_{16}$	CCACCCCAATTAgATTAgCTC	ggATggTTTgTgACAATTTgC	58	Adapted for this study from Millis (2000)	HM448902
Tv_B38.1	$(CA)_3GA(CA)_{13}$	CACgTTgTCTCCCACCCC	CCAgTAAAgATTTggAAgAgg	58	Adapted for this study from Zenger and Johnson (2001)	HM448903

were amplified with one locus per reaction. Cycling was performed in an Eppendorf Mastercycler with an initial denaturation of 4 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 20 s at the annealing temperature (Table 1), 30 s at 72 °C, and a final extension of 4 min at 72 °C. Amplicons from the nine loci were pooled into two batches and genotyping performed on a CEQ™8000 with a 60–420 bp or 60–640 bp size standard as appropriate (Beckman Coulter, Brea, CA, USA). Results were analysed with the CEQ 8000 Genetic Analysis System v 8.0.52 (Beckman Coulter).

Data analysis

Locus and population statistics. We genotyped 1790 individuals but included in our final analysis only the 1605 individuals for which genotypes were obtained from at least five loci (mean = 7.9 loci/individual). We tested for linkage disequilibrium within sites using GENEPOP 4.2 (Rousset 2008) and for departures from Hardy–Weinberg Equilibrium using Chi-square tests within the R-package POPGENREPORT (Adamack & Gruber 2014). All multiple comparison tests were conducted using a strict Bonferroni correction.

Genetic diversity within samples from each site was calculated as the mean number of alleles per locus, the observed heterozygosity (H_O) and the heterozygosity expected under conditions of Hardy–Weinberg equilibrium (H_E) using the R-packages POPGENREPORT (Adamack & Gruber 2014), and ADEGENET (Jombart 2008). Nei's G_{ST} (Nei 1973; Nei & Chesser 1983) was determined between all pairwise combinations of sampling sites using the R-package MMOD (Winter 2012).

Population structure of possums. We tested for population genetic structure using a suite of approaches as suggested by Blair *et al.* (2012) applying two Bayesian cluster approaches and a spatial principal components analysis. Initially, we applied Bayesian cluster analysis as implemented in STRUCTURE 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003) to the multilocus genotype data with and without the LOCPRIOR option. We assumed an admixture ancestry model with correlated allele frequencies and ran STRUCTURE for K equal to one to 10, with 20 replicates being run for each level of K. For each replicate, we ran 10^5 burn-in steps followed by 2×10^5 MCMC steps. The optimal number of clusters was determined using the Evanno method (Evanno *et al.* 2005) as implemented by STRUCTURE HARVESTER (Earl & Vonholdt 2012). To confirm the optimal number of clusters as determined by STRUCTURE HARVESTER, we used CLUMPP (Jakobsson & Rosenberg 2007) to align cluster labels across replicate runs for $K = 2$ to optimal K and then compared barplots of cluster assignments across

replicates using *R* to ensure that assignments were consistent across runs and that there were no 'ghost clusters' (Durand *et al.* 2009).

We also used spatial Bayesian cluster analysis (BMY admixture model; Durand *et al.* 2009) as implemented in the program TESS 2.3.1 (Chen *et al.* 2007). TESS was run for $K = 2$ to 10 clusters, with 20 replicates for each value of K and 2×10^5 total sweeps of which 10^5 were burn-in sweeps for each replicate. The optimal number of clusters was determined by identifying the K at which DIC values plateaued and by inspecting barplots of cluster assignments for $K = 2$ to the K at which DIC values plateaued across replicates to confirm that assignments had stabilized. CLUMPP (Jakobsson & Rosenberg 2007) was used to align cluster labels across replicates while population assignment barplots were generated using *R*. Once the optimal number of clusters was determined, we performed 400 replicates for the optimal number of K and retained the 10% of replicates (40 replicates) with the lowest DIC values. Cluster labels were again aligned using CLUMPP, and then, each individual's cluster assignment was averaged across replicates to produce a consensus cluster assignment for each individual. Consensus cluster assignments were then mapped.

Finally, we used spatial principal component analysis (sPCA; Jombart *et al.* 2008) implemented in the R-package ADEGENET (Jombart 2008; Jombart & Ahmed 2011) to spatially model the population genetic structure identified using georeferenced multilocus genotypes (Jombart *et al.* 2008). This approach is highly suitable for analyses of complex or cryptic genetic structures, such as hybrid zones, because it does not require assumptions of Hardy–Weinberg and linkage equilibrium. Two separate sPCA analyses were applied to our possum data set. The first sPCA analysis was applied to individual genotypes, allowing us to view individual variability in assignments. The second sPCA analysis was applied to grouped genotypes for each sampling site, which reduces the effect of individual variability, but provides a clearer, more easily interpretable result. For the individual analysis, we used a distance-based connection network (minimum distance between neighbours = 0 m, maximum Euclidean distance = 5000 m based upon the approximate mean dispersal distance from Cowan *et al.* 1996) as possum sampling locations were clumped. For the grouped analysis, we used a Gabriel graph connection network which eliminates many of the overly long connections seen with connection networks based on Delaunay triangulation (Legendre & Legendre 1998 p. 752–756). sPCA eigenvalues were tested for global (patches, clines and intermediates) and local (strong genetic differences between neighbours) spatial structure, separately for each analysis, using Monte Carlo tests (10^4 permutations). In our sPCA analysis for individuals, we excluded one individual from the sPCA as it was found to be an extreme outlier having three unique alleles, one of which was homozygous at one locus while the two other unique alleles were heterozygous at a second locus. These unique alleles did not appear to be the result of mis-scoring or a genotyping error.

Assessing the impact of rivers on dispersal. The effects of rivers and geographic (Euclidean) distance on differentiation between sampling sites was investigated using a three-step process. First, sampling sites were subdivided into possum population groupings based upon the STRUCTURE results using sampling location as a prior (with LOCPRIOR). Second, as males and females are known to exhibit differences in dispersal, we tested for differences in the pattern of spatial autocorrelation between male and female possums for each population grouping using the spatial autocorrelation heterogeneity test provided in GENALEX 6.53b (Peakall & Smouse 2006, 2012; Banks & Peakall 2012). Third, a combination of Mantel and partial Mantel tests using the R-package VEGAN (Oksanen *et al.* 2013) were performed to test for the effects of rivers and geographic distance on differentiation between sampling sites (Cushman *et al.* 2006, 2013). Separate sets of Mantel and partial Mantel tests were performed for each population grouping or where necessary subgrouping (males and females) based upon the results of the tests for heterogeneity in spatial autocorrelation for males and females. Two Mantel tests (Nei's pairwise Gst adjusted for isolation by distance (Rousset 1997) correlated with geographic distance or number of river crossings) and reciprocal partial Mantel tests (Nei's pairwise Gst correlated with geographic distance or number of river crossings controlling for number of river crossings or geographic distance, respectively) were performed for each population grouping (by sex), with 10 000 permutations. Reciprocal partial Mantel tests (Cushman *et al.* 2013) were used to minimize the risk of type I errors (Guillot & Rousset 2013). Distance matrices used in the Mantel and partial Mantel tests were calculated within *R* or by hand (number of river crossings described later). Nei's pairwise G_{ST} distance matrices for each population grouping or subgroups were calculated using the package MMOD (Winter 2012) in *R*. The following rules were used to determine the number of river crossings between pairs of sites: First, the number of river crossings was set to 0 if it were possible for individuals to travel from one site to another without crossing a broad river segment or around the headwaters of a river system (e.g. between river basins). Second, we set the number of river crossings to the least number of rivers that needed to be

crossed to move between pairs of sites where sites were separated by at least one broad river segment. Under this rule, sites 4, 6, 7 and 14 were treated as being within the headwaters of the river systems, and thus, the number of river crossings between each of these sites was set to 0. Further, we treated sites 14, 17, 21 and 22 as being within the same river basin and requiring 0 river crossings to travel between one another. Similarly, sites 4, 6, 7, 10, 11, 15, 20 and 32 were treated as being within the same river basin requiring 0 river crossings to move between sites.

Finally, we used a least-cost path approach to test for the impact of the key landscape features (rivers – including bridges – and forest and scrub cover) identified by Etherington *et al.* (2014) as affecting genetic connectivity of possums among sampling locations in the area. To avoid confounding landscape feature effects with interactions between possums of different origins (Tasmanian, mainland or an admixture) and to ensure sufficient numbers of sites, forests and rivers, we focussed our analysis of these features within the single largest collection of sites identified as a single cluster in STRUCTURE (where sampling location was used as a prior with LOCPRIOR). For a least-cost path analysis that includes all taxonomic entities, and a more detailed description on the creation of the layers used please refer to Etherington *et al.*(2014). In our analysis, we used raster layers of rivers (including information on bridges) and tree and scrub cover derived from GIS map layers (1:50 000 scale) provided by Land Information New Zealand (http://www.linz.govt.nz) and converted to raster by Etherington *et al.* (2014). To combine the raster layers, we scaled tree and scrub values (% of land cover) inversely such that higher resistance effects resulted from low tree and scrub cover and minimum resistance occurred at 100% forest and scrub cover. For the river and bridges raster, we used a GIS layer derived from a digital elevation model of the river drainage data set in which the main rivers were represented as a stream order greater than four. Here, each value in a raster cell was calculated as $\frac{1}{9-\text{river order}}$. The maximum river order was 8, and hence, the maximum value in a cell is 1, and the effect is scaled in such a way that the lower the river order, the lower the resistance value. If a bridge was present in a cell, we set the resistance value to zero under the assumption that bridges negated the barrier provided by a river. To study the effect of each layer separately, we weighted the values of each cell of each layer with levels of 0.1, 1, 5, 10, 50, 100, 500 and 1000 and then calculated least-cost paths using the four nearest neighbours rule, separately for each of the raster layers using the newly developed *R* function LANDGENREPORT in the package POPGENREPORT (Adamack & Gruber 2014). Application of the four nearest neighbours rule avoids geometric artefacts at the bends of linear structures such as rivers. The resulting pairwise least-cost distance matrix between sample sites was then compared with a pairwise genetic distance matrix derived using Nei's G_{ST} and corrected for isolation by distance through the inclusion of a simple Euclidean distance matrix. We used multiple matrix regression with randomization (MMRR; Wang 2013) to identify the best-fit resistance level for each raster layer. To further identify the amount of variation that could be explained by 'rivers including bridges' and 'forest and scrub cover', we combined the two raster layers, setting their respective resistance weights to all combinations of the best-fit resistance level previously obtained ±2 resistance levels. Using this approach, resistance ranged from 1, 5, 10, 20, 50 for rivers including bridges and 10, 50, 100, 200, 500 for forest and scrub cover making a total of 25 combinations. We again used multiple matrix regression with randomization to determine the best fit of the combination of resistance levels.

Results

Locus and population statistics

The number of alleles and heterozygosity level observed at each locus at the Hawke's Bay site was similar to those recorded in other studies of New Zealand and Australian possums where the same loci were used (Table 2). Genetic differentiation was low among all 31 sites with an average Nei's G_{ST} over all loci of 0.039 (95% CI = 0.037–0.042).

Population structure

Tests for linkage disequilibrium within sites revealed disequilibrium in only a single combination of loci and site (Tv16 and TvM1; site 34; $P < 0.0001$) so we consider all loci to be in equilibrium within sites. Similarly, only a limited number of combinations of locus, site and sex (nine for females and five for males out of 558 total combinations) were found to depart from Hardy–Weinberg Equilibrium. Analysis with the program STRUCTURE 2.3.4 using the LOCPRIOR model revealed two inferred population clusters ($K = 2$; Fig. 2, Table S1; Figs S1, S2, Supporting information) among the 31 sample locations. The model identifies a clear and geographically well-defined gradation of genotypes from predominantly (>90%) red histograms at sites 1 and 2 (Fig. 2) in the south west to predominantly (>90%) blue histograms in the north east (sites 22, 23, 27, 28, 30 to 35; Fig. 2). In between these sites, individuals display admixture, with proximity to each of the parent genotype populations

Table 2 Genetic variability measures for nine microsatellite loci. H_E, expected heterozygosity under Hardy–Weinberg equilibrium

Locus	No. of alleles*	No. of alleles NZ/Aus†	H_E*	H_E† NZ/Aus	Allele size range†,‡,§	Allele size range present study
Tv16	15	6.7 (1.2)	0.78	0.79/0.81	110–148	110–148
Tv19	21	6.6(1.3)	0.77	0.71/0.90	224–296	224–296
Tv53	19	10.5(2.3)	0.87	0.80/0.84	219–275	219–275
Tv58	10	5.5(1.7)	0.52	0.76/0.84	121–157	133–157
Tv64	19	7.2(1.8)	0.76	0.83/0.88	138–200	138–200
Tv5.64	12	4.2(1.1)	0.39	—	221–303	221–303
Tv_PnMs16	15	4.8(2.0)	0.60	—	220–254	220–254
Tv_B38.1	15	6.1(1.7)	0.75	—	61–97	61–97
TvM1	13	6.4(1.2)	0.78	0.73/0.77	219–251	219–251

–, data not available.
*Data from the current study.
†Data from Taylor & Cooper (1998) collected from populations around New Zealand.
‡Data from a study of a single population at Hawke's Bay by Taylor *et al.* (2000).
§Study by Lam *et al.* (2000).

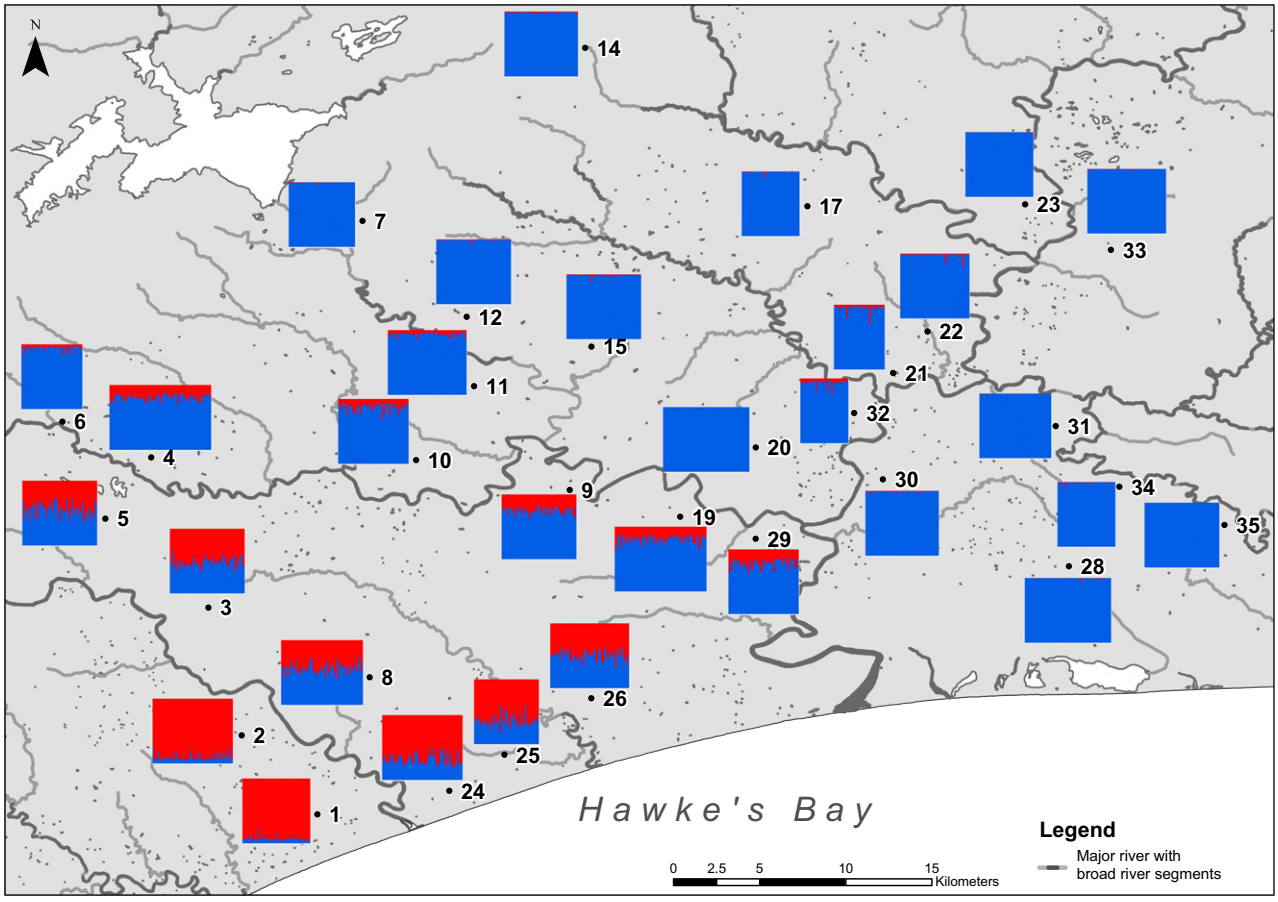

Fig. 2 STRUCTURE cluster assignment barplots for each sample site using sampling locations (LOCPRIOR) as a prior assumption for cluster assignments. STRUCTURE HARVESTER indicated that a structure of $K = 2$, is consistent across replicates. Note: red and blue genotypes here correspond to 'red' and 'blue' groups referred to in the text.

influencing the level of admixture. The two clusters correspond approximately to the distribution of coat colour in the area (Fig. 3) with grey fur more likely and brown fur less likely the more strongly a site was inferred to be the red cluster (R^2 = 0.81, P = 4.744e-12 and R^2 = 0.32, P = 0.0005542) and reflect the known release history of possums of Tasmanian origin (presumed *T. v. fuliginosus*; blue histograms) to the north and east and mainland origin possums (presumed *T. v. vulpecula*; red histograms) to the southwest (Figs. 1-3). The admixed sites form a zone that appears to be greater than 25 km at its widest point and to extend for over 35 km bounded by the limits of our sampling and between the sites of predominantly parental genotypes (Fig. 2). When the admixture model of STRUCTURE (no LOCPRIOR) is applied, the zone of admixture remains, but broadens, with introgression of the *T. v. vulpecula* type appearing in most of the *T. v. fuliginosis* dominated sites (Figs S3-S5, Supporting information). Outputs from the program TESS also indicate a zone of hybridization (Fig. S6, Supporting information); however, plots of DIC values vs. K values, cluster assignment barplots across replicates, and final tessellation plots from TESS (data not shown) suggest the optimal number of clusters to be four rather than two (Fig. S7, Supporting information). Comparison of Fig. S7 (Supporting information) with similar figures for K = 2 and K = 3 (not shown) indicate that the orange and green groups occupy a zone of hybridization similar to that identified in STRUCTURE.

Results of both sPCA analyses (individuals and grouped by site) indicate that there was significant global spatial structure (individuals $P = 3 \times 10^{-4}$; grouped by site $P = 2 \times 10^{-4}$), but no local spatial structure (individuals P = 0.8741; grouped by site P = 0.9989). We identified a single interpretable eigenvalue for global structure (Fig. S8, Supporting information) in the individual analysis, which was used to produce an interpolated map of individual scores (Fig. 4). We interpret the eigenvalue as indicative of the genetic origin of the possum: Australian

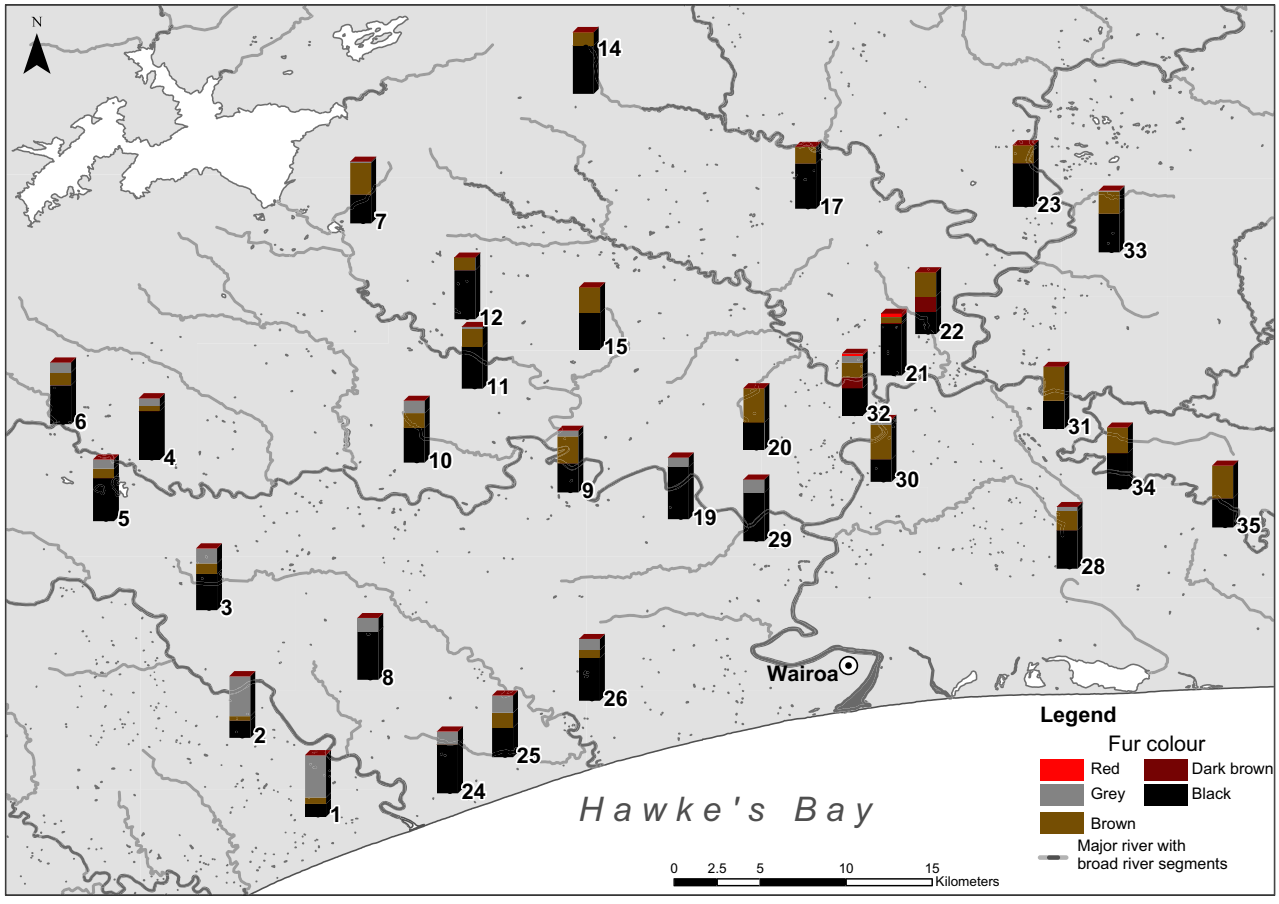

Fig. 3 Observed possum fur colour by sampling site. Each stacked barplot shows the proportion of the possums observed at a sampling site whose fur colour was (from bottom of stack to the top) red, grey, dark brown, brown or black. Proportions summed to one at each site. Numbers = sample size at each site.

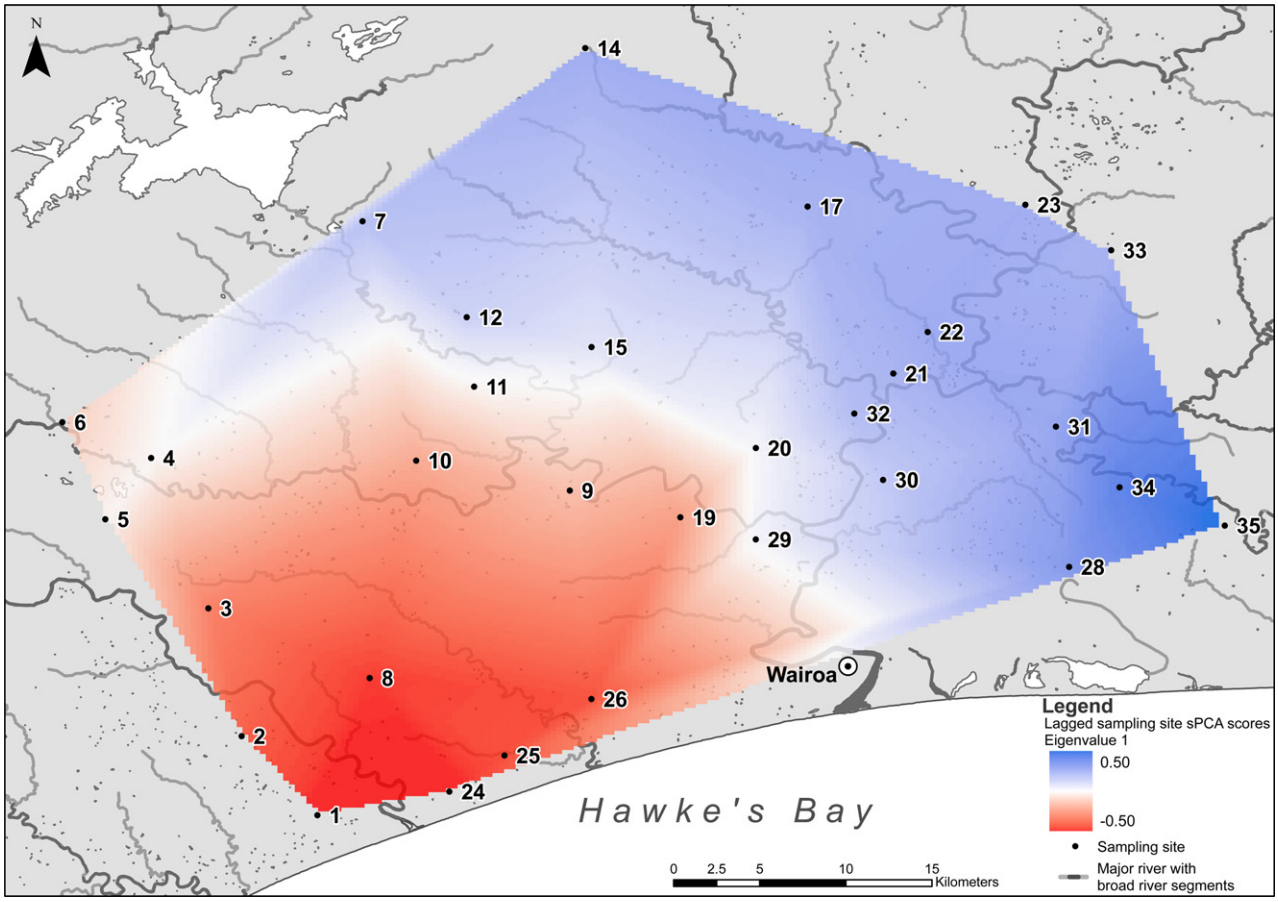

Fig. 4 Interpolated map of individual sPCA scores for the first eigenvalue. The red and blue colours represent the distribution of individuals most closely aligned with possums of mainland Australian origin (grey coat colour) and Tasmanian (black and red coat colour) origin, respectively.

mainland, Tasmanian or a hybrid of the two as Fig. 4 is spatially consistent with the STRUCTURE (Fig. 2 and Fig. S3) and TESS (Fig. S6) results that indicate a genetic origin to the structure. Two eigenvalues were identified as interpretable in the grouped by sites analysis (Fig. S9, Supporting information). Like the individual analysis, we interpret the first eigenvalue as indicating the genetic origin of the possum (Fig. S10, Supporting information), while the second eigenvalue indicates the degree of hybridization of the possum (Fig. S11, Supporting information). Spatial maps of the sPCA scores for individuals and groups were similar to one another (Fig. 4; Fig. S10, Supporting information), with both indicating two main groupings of possums whose spatial structures were generally consistent with those indicated by both sets of STRUCTURE runs (Fig. 2 and Fig. S3, Supporting information) and TESS (Fig. S6, Supporting information). Further, the maps show a sharp transition between the two main groupings that appears to coincide with the Wairoa River at its lower reaches but departing from it further upstream.

Rivers as barriers

Given the distinct genetic structuring seen between the subspecies of possum and the likely input that that underlying structure would have on intersite genetic comparisons, we confined our analyses of the impact of rivers on population genetic structure to a single genetic unit. Specifically, we chose the largest genetic grouping (blue histograms; Fig. 2; sites 22, 23, 27, 28, 30–35) identified through our STRUCTURE runs using sampling sites as a prior. Comparisons of the patterns of spatial autocorrelation for male and female possums within this 'blue' group using heterogeneity tests (Banks & Peakall 2012) revealed significant heterogeneity between males and females ($\Omega = 89.43$, $P = 0.001$). We also identified spatial autocorrelation over 37–40 km. Simple Mantel tests showed that pairwise estimates of Nei's Gst were spatially correlated with Euclidean distance for female 'blue' possums but not males while both sexes were significantly correlated with the numbers of river crossings (Table 3). However, when distance is controlled for, the

number of crossing remained significant for males but ceased to be significant for females (Table 3) indicating a sex bias in dispersal. It appears clear that for males the number of river crossings between sites is more important than Euclidean distance in controlling genetic differentiation while for females it is the Euclidean distance that is important. We interpret these results as indicating that as males naturally disperse further than females (on average), they are more likely than females to encounter a river and have their dispersal affected by such barriers. Similarly, for blue populations, we found that the best least-cost path scenario for the 'rivers including bridges' scenario was 10 ($R^2 = 0.18$; F = 22.25; $P < 0.003$; Table 4), while for the 'forests and scrub cover layer' only, the best resistance scenario was for a friction of 100 ($R^2 = 0.33$; F = 49.86; $P < 0.001$; Table 5). When the forest and scrub cover and rivers including bridges layers were combined, we found that the best friction values were five for the rivers including bridges and 200 for forest and scrub cover ($R^2 = 0.32$; F = 47.78; $P < 0.001$; Table 6). We note that rivers, including bridges had a negative relationship with differentiation using the multiple matrix regression with randomization analysis, while forest and scrub cover had a positive relationship with differentiation. This indicates that rivers were a barrier to connectivity for possums while more tree and scrub cover resulted in increased connectivity between sampling sites.

Discussion

Our analyses reveal a surprising distribution of genetic variation among possums in the Hawke's Bay region of New Zealand. We have identified three distinctive

Table 3 Mantel tests for correlation between pairwise values (among sampling sites) of Nei's Gst and the Euclidean distance (Distance) and number of river crossings (Crossings) between sampling sites and reciprocal partial testing for correlations between Nei's Gst and Euclidean distance or number of river crossings while controlling for number of river crossings or Euclidean distance, respectively. Bold-faced values indicate significance at $P = 0.05$

	Mantel				Partial Mantel			
	Nei's Gst ~ Distance		Nei's Gst ~ Crossings		Nei's Gst ~ Distance | Crossings		Nei's Gst ~ Crossings | Distance	
Sex	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Male	0.17	0.0679	0.42	**0.0004**	0.032	0.3863	0.39	**0.0028**
Female	0.36	**0.0019**	0.27	**0.0291**	0.30	**0.0195**	0.17	0.1287

Table 4 Multiple matrix regression with randomization summary table for the least-cost path analysis performed using only the rivers including bridges raster file with river resistance set to 10 and non-river resistances set to 0. The analysis is for the blue populations only

Layer	Coefficient	*t*	*P*-value (*t*)	F	*P*-value (F)	R^2
River (resistance = 10)	2.73×10^{-7}	2.45	0.016	22.25	0.003	0.18
Euclidean distance	-6.17×10^{-9}	−0.039	0.98			
Intercept	1.02×10^{-2}	10.48	1.00			

Table 5 Multiple matrix regression with randomization summary table for the least-cost path analysis performed using only the forest and scrub cover raster file with forest and scrub cover resistance set to 100 and non-forest and scrub cover resistance set to 0. The analysis is for the blue populations only

Layer	Coefficient	*t*	*P*-value (*t*)	F	*P*-value (F)	R^2
Forests (resistance = 100)	2.42×10^{-8}	6.74	0.004	49.86	0.001	0.33
Euclidean distance	1.56×10^{-7}	3.95	0.030			
Intercept	7.55×10^{-3}	8.08	1.00			

Table 6 Multiple matrix regression with randomization summary table for the least-cost path analysis performed using the combined rivers including bridges and forest and shrub cover raster files with forest and shrub cover resistance set to 200, and river resistance set to 5 and all other resistances set to 0. The analysis is for the blue populations only

Layer	Coefficient	*t*	*P*-value (*t*)	F	*P*-value (F)	R^2
Rivers (resistance = 5)	1.23×10^{-8}	6.48	0.004	47.78	0.001	0.32
Tree coverage (resistance = 200)						
Euclidean distance	1.76×10^{-7}	4.54	0.012			
Intercept	7.64×10^{-3}	8.13	1.00			

genetic groups: one composed almost entirely of animals most likely from a mainland Australian origin (*T. v. vulpecula*), one composed almost entirely of animals most likely from a Tasmanian origin (*T. v. fuliginosis*), and one in which those two subspecies are introgressed in a clinal manner forming a hybrid zone as defined by Barton & Hewitt (1985). The parental genetic groups are geographically distributed in accordance with the documented history of the introductions of two subspecies and with the current distribution of coat colours such that, predominantly grey (mainland origin possums) are present in the south and predominantly black or brown possums (Tasmanian origin) are in the north and east with a zone of hybridization in between them of mixed colours (compare Fig. 3). Contact between the two parental genetic clusters has occurred as *T. v. vulpecula* was released near the current contact zone in 1936 and is likely to have existed for many decades.

Introgression occurs on either side of the hybrid zone; however, the extent of the introgression evident is influenced by the method of analysis used, suggesting that more data localized to the hybrid zone will be required to obtain a sufficiently precise understanding of its extent and function. The sPCA shows a steep cline from *T. v. vulpecula* ('red') to *T. v. fuliginosis* ('blue') that may be only a few kilometres wide and cannot be attributed to the barrier effect of rivers alone as their geometry does not follow the hybrid zone (Fig. 4). Hybridization between F1 hybrids and parental genotypes appears likely, as the probability of an individual containing a parental genotype declines with distance from the parental sites (either 'red' or 'blue' sites). Nevertheless, it appears that the hybrid zone functions to some extent as a barrier to the dispersal of full parental genotypes. This is indicative of some form of incompatibility between the two parental genetic clusters that promotes assortative mating at the point of contact.

It is evident that the major rivers in the region act as partial barriers to possums while trees and scrub cover promote interaction. As such it is possible that the presence of rivers has contributed to the formation of the hybrid zone in the region, by limiting dispersal sufficiently to enable the hybrid zone to stabilize more readily than might otherwise occur. Nevertheless, even if this were a factor in hybrid zone formation, it is not sufficient to explain the existence of the zone in its current position. Although the average dispersal distance for individual possums is estimated at about 5 km (Cowan *et al.* 1996), dispersing possums have been observed to cover distances of several tens of kilometres in a short time, with 41 km being the largest dispersal distance recorded (Cowan & Clout 2000) and to cross major rivers (Cowan 2001), making possible the opportunity for introgression in the current study area (Fig. S12, Supporting information) using the relatively contiguous tracts of trees and scrub (Fig. S13, Supporting information). It thus appears likely that populations of *T. v. vulpecula* made contact with populations of *T. v. fuliginosus* relatively soon after their introduction to the area in 1936, at a site approximately 45 km from the current contact zone (Fig. 1), suggesting that the contact zone has existed for some decades. In essence, the hybrid zone appears to be biological in formation even if the distribution of major rivers in between the points at which the possums were released has influenced the positioning of the zone.

Overall, our data suggest that there are two distinct genetic entities in the region that are capable of interbreeding and forming hybrids, but which appear to exhibit assortative mating at the point of contact. In effect, these genetic clusters align to forms that have been previously designated as separate subspecies in allopatry but which, through inadvertent biological invasion, now exist in sympatry.

Maintenance of the hybrid zone

We have sampled the Hawke's Bay hybrid zone at one point in time. We cannot, with the data available, determine whether the hybrid zone is also a tension zone and maintained by selection and therefore stable with respect to local environmental conditions (Key 1968), or

one that is transitory and likely to break down into a broader zone of introgression that eventually will not be able to be defined. The low levels of G_{ST} between the 'blue' and 'red' genetic clusters (mean = 0.029) suggest that the incompatibility responsible for the formation of the hybrid zone, either in terms of viability of offspring or aspects of species recognition or mating systems, could be affected by relatively few loci. An obvious candidate for such incompatibility is the MHC complex which can affect species recognition and New Zealand possums are known to show geographical variation at class II MHC loci (Holland *et al.* 2008). Further investigation through either targeting genes such as those associated with MHC or a broader level genomic approach is required to determine the biological basis for this phenomenon.

Implications for managing populations of this invasive species

Our findings have significant implications for the way in which New Zealand's most economically important pest is managed because multiple interactions between the two subspecies are likely. Surveys dating back to the 1970s and earlier suggest that there are more than 90 sites on the North Island of New Zealand alone that carry mixed populations of *T. v. vulpecula* and *T. v. fuliginosus* (L. T. Pracy and J. D. Coleman unpub. data), raising the possibility that there are many contact zones between these two subspecies. If hybrid zones are formed as a regular construction of these contact zones, then possums in New Zealand are likely to exist as a complex mosaic of subspecies, with potentially hundreds of hybrid zones across the landscape. It is also likely that *T. v. vulpecula* from the Australian mainland were originally sourced from more than one locality creating the opportunity for multiple hybrid forms.

Hybrid zones that act as barriers to dispersal, as appears to be the case in the present study, may inhibit dispersal and therefore affect the way in which possums use the landscape. As possums are the main wildlife vector for bovine tuberculosis (Caley *et al.* 1999), hereto cryptic hybrid zones may act to lessen the threat of bovine Tb transmission in some parts of the country. Conversely, disruption of hybrid zones through broad control measures, such as widespread baiting, may in fact increase the rates of dispersal through what were formerly hybrid zones.

Evolutionary pathways for T. vulpecula in New Zealand

The available evidence from studies of control suggests that well-managed poisoning and trapping will cause a substantial (≥80%) reduction in the density of possums and that recolonization by possums of buffer zones between infected and noninfected cattle will occur gradually, initially through possums in adjacent areas extending their home ranges (Efford *et al.* 2000) or by dispersal from more distant areas (Ji *et al.* 2001). The rate at which recolonization occurs will depend on multiple factors such as habitat type, distance to the nearest suitable uncontrolled source of possums and local topography (Cowan *et al.* 1996, 1997). The observed difference in susceptibility between the two subspecies to the poison 1080 (Henderson *et al.* 1999) has the potential to disrupt existing hybrid zones or contribute to their redistribution over time by causing differential mortality among the genetic clusters particularly without optimal baiting dosages (Frampton *et al.* 1999). However, the potential for this to occur has been reduced substantially in New Zealand through a policy of toxic loading by colour morph. It is also conceivable that the development of resistance to 1080 such as that seen in Australian rabbits (Twigg *et al.* 2002) could be hastened by the formation of hybrids among different genetic forms of possums, particularly when some forms show lower susceptibility than others. More needs to be known about possible mechanisms for the potential for such resistance to occur.

Conclusions

Our population genetic analyses reveal a hitherto unknown hybrid zone among introduced possums in New Zealand that could only have been formed since 1936. There appears to be a biological basis for the hybrid zone, but it is also possible that local geography, particularly the existence of large rivers, has contributed to its formation.

It is likely that this is one of many such hybrid zones formed around New Zealand. The existence of such zones could have a number of important implications for the way in which possum populations are managed, particularly when attempting to minimize the rate of spread of bovine TB through transfer by possums to noninfected areas. Most importantly, our data suggest that multiple hybrid zones may act to reduce the rate at which possums disperse across some landscapes by providing a hitherto unrecognized biological barrier. Conversely, intense baiting across a hybrid zone may in fact promote dispersal by breaking down that biological barrier enabling more rapid dispersal across the baited area. More experimental work is required to determine how such hybrid zones may affect possum dispersal in relation to topography and control through baiting. The presence of two forms of possum in New Zealand has resulted in unique genetic combinations that do not

occur in their native range and opens the door to the rapid evolution of new forms.

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S.D.S. and P.C. conceived and designed the research project with N.A. P.C. coordinated the sample collection. N.A. genotyped the samples and developed new loci with A.J.M., A.T.A. and B.G. analysed the data. S.D.S., P.C., N.A., A.T.A., A.J.M. and B.G. wrote the article.

Data accessibility

DNA sequences GenBank Accessions nos HM448904, HM448905, HM448906, HM448902, HM448903; Sample locations and microsatellite data have been lodged with DRYAD (doi:10.5061/dryad.8364h Data files: possum_genotypes).

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Ln(Pr(X|K) for each replicate of *K* (replicates = 20) for *K* = 1 to 10 for the LOCPRIOR STRUCTURE run.

Fig. S2 Delta K for each value of *K* = 2 to 10 as determined using the Evanno method, as implemented in STRUCTURE HARVESTER, for the LOCPRIOR STRUCTURE runs

Fig. S3 STRUCTURE cluster assignment barplots subset by sampling site and placed at the approximate location of the sampling site for the STRUCTURE run without LOCPRIOR. STRUCTURE HARVESTER indicated that a structure of *K* = 2, was consistent across replicates. Colours as for Fig. 2.

Fig. S4 Ln(Pr(X|K) for each replicate of *K* (replicates = 20) for *K* = 1 to 10 for the STRUCTURE run without LOCPRIOR.

Fig. S5 Delta K for each value of *K* = 2 to 10 as determined using the Evanno method as implemented in STRUCTURE HARVESTER for the STRUCTURE runs without LOCPRIOR

Fig. S6 TESS cluster assignment barplots subset by sampling site and placed at the approximate location of the sampling site for the TESS run. Examination of TESS diagnostics (plots of DIC values vs. *K*, stability of replicate barplots, and the final TESS tessellation maps) indicated that the optimal *K* was 4 groups.

Fig. S7 Deviance Information Criterion (DIC) plot for each replicate of *K* (replicates = 20) for *K* = 2 to 10.

Fig. S8 Spatial and variance components of the eigenvalues for the individual sPCA. The maximum attainable variance by a linear combination of alleles from an ordinary PCA is indicated by the vertical dashed line on the right while the two horizontal vertical dashed lines indicate the range of variation of Moran's I given the spatial weighting matrix that was used (as described in Jombart 2013).

Fig. S9 Spatial and variance components of the eigenvalues for the grouped by sites sPCA. The maximum attainable variance by a linear combination of alleles from an ordinary PCA is indicated by the vertical dashed line on the right while the two horizontal vertical dashed lines indicate the range of variation of Moran's I given the spatial weighting matrix that was used (as described in Jombart 2013)

Fig. S10 Interpolated map of grouped by site sPCA scores for the first eigenvalue. The observed spatial pattern of sPCA scores for the first eigenvalue of the grouped by site sPCA appears to be consistent with the spatial pattern observed for the first eigenvalue of the individual sPCA.

Fig. S11 Interpolated map of grouped by site sPCA scores for the second eigenvalue. This eigenvalue appears to be associated with the degree of hybridization of individuals with darker regions being more hybridized than paler areas.

Fig. S12 Possum sampling sites in the study area overlain by the 'rivers including bridges' resistance surface. Resistance was modelled in such a way that the higher the stream order the higher is the resistance. Bridges were modelled as gaps (zero resistance) that can be crossed without resistance and are represented on the map by minute gaps in the rivers.

Fig. S13 Possum sampling sites in the study area overlain by the 'forest and scrub cover' resistance surface. Darker colours represent higher resistance values but lower tree and scrub cover.

Table S1 Inferred membership proportions for the predefined sample locations to the two population clusters identified during the LOCPRIOR STRUCTURE analysis. Sampling sites were assigned to a population unit ('Red' or 'Blue') if their inferred population cluster were greater than 0.8 for 'red' or 'blue' respectively. 'Red' = likely *T. v. vulpecula* in origin, 'Blue' = likely *T. v. fuliginosis* in origin. Sites that were not classed as either 'Red' or 'Blue' were considered to be hybrids the two subspecies. SD = standard deviation.