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Anthropogenic cause of range shifts and gene flow between two grasshopper species revealed by environmental modelling, geometric morphometrics and population genetics

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Abstract. 1. The range of a species is controlled by biotic and abiotic factors; both could have changed recently due to human activity.

2. We used environmental modelling, morphometric and genetic data to interpret ecological responses at the species boundary of a pair of New Zealand grasshoppers with very different ranges; one widespread (*Phaulacridium marginale*) and one restricted to semi-arid central/southern South Island (*Phaulacridium otagoense*).

3. Climate- and habitat-based distribution models for grasshoppers in the past (last glacial maximum), present and future (2070), in concert with modelling of vegetation patterns imply range and demographic expansion of *P. marginale* and stability of *P. otagoense*.

4. mtDNA sequence revealed four main lineages with pronounced differences in genetic diversity and geographical range. The widespread lineage associated with *P. marginale* revealed a signature of range expansion but regionally restricted lineages were geographically structured at a fine scale. Within the narrow geographical range of *P. otagoense*, three mtDNA lineages resulted in high diversity, more typical of large stable populations.

5. Geometric analysis of pronotum shape identified individuals from a region of sympatry with mixed characteristics. Mismatch of phenotype, mtDNA lineage and nuclear DNA sequence indicates introgression between grasshopper species now in contact. This appears to be accompanied by *P. otagoense* range reduction through ecological competition.

6. Deforestation by people starting $\sim$800 years ago best explains range change and resulting hybridisation of these grasshoppers. Anthropogenic habitat modification can have indirect consequences on insect biodiversity and conservation by enabling introgression between formerly separate populations and species.

Key words. Climate change, global warming, habitat modification, hybridisation, last glacial maximum, niche models.

Introduction

The major influence of climate on species distribution and abundance has been apparent since the introduction of phylogeographic tools (Avise *et al.*, 1987) that revealed the population genetic signature of expanding and contracting plant and animal ranges (e.g. Taberlet *et al.*, 1998; Hewitt, 2004; Excoffier *et al.*, 2009). More recently, the same approach has been directed at documenting and predicting outcomes of climate change in the Anthropocene (e.g. Robinet & Roques, 2010; Alsos *et al.*, 2012; Andrew *et al.*, 2013; Pauls *et al.*, 2013; Razgour *et al.*, 2015).

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Recent rapid range shifts may have an overriding effect on the extent and distribution of intra-specific genetic variation (e.g. Excoffier *et al*., 2009; Garroway *et al*., 2011; Bubac & Spellman, 2016), that is most often apparent when comparing closely related species (e.g. Lewis & Crawford, 1995; Chen *et al*., 2007; Blair *et al*., 2014; Krosby *et al*., 2015). Different histories of range change create different patterns of genetic structure in related species (Hewitt, 2004). Species pairs provide valuable insight on evolutionary ecology because they allow comparison of population responses at the species boundary (e.g. Mathews *et al*., 2002; Acevedo *et al*., 2012; Dawson, 2012; Bulgarella *et al*., 2014). Closely related lineages frequently have adjacent ranges with differing environmental characteristics leading to ecological adaptation and restricted niche breadth (Endler, 1977; Peers *et al*., 2013), so that an interplay between gene flow and selection on adaptive traits exists unless assortative mating evolves (Drès & Mallet, 2002; Emelianov *et al*., 2004). Shifts in resource availability associated with changing global environmental conditions could drive range changes that alter gene flow equilibria (Lenormand, 2002). In turn, this may redirect the outcome of current competitive interactions (Acevedo *et al*., 2012; Bulgarella *et al*., 2014), especially where these are influenced by range overlap (Krosby *et al*., 2015). The detection of species responses to past and future environmental change is of increasing interest and necessity as the consequences of anthropogenic habitat modification becomes better understood (Hellmann *et al*., 2012; Razgour *et al*., 2013; Wisz *et al*., 2013).

Here, we considered the situation for a sister pair of endemic short-horned grasshoppers that are the only representatives of the genus *Phaulacridium* (Key, 1992) in New Zealand. They live primarily in low elevation grassland, with *Phaulacridium marginale* (Walker, 1870) distributed in mesic habitats through New Zealand and many near-shore islands (Westerman & Ritchie, 1984), and *Phaulacridium otagoense* (Westerman & Ritchie, 1984) restricted to a limited semiarid habitat in central South Island. This distribution suggests species ecology linked to environmental conditions associated with climate, and the potential for different responses to climate change and human modification of the landscape. The contrasting distributions of the two species (widespread vs. restricted) furnish a prediction of contrasting genetic diversity due to population size variation and isolation by distance (Wright, 1943; Slatkin, 1993; Charlesworth, 2009), and a mismatch of mtDNA diversity and spatial distribution has been reported in these grasshoppers (Goldberg *et al*., 2015). If, as it appears from low genetic diversity, *P. marginale* has recently expanded its spatial range, what change drove this response? Possibly *P. otagoense* formerly had a wider range during the last glacial maximum (LGM; ~20 kya) when drier conditions were more widespread, but has now retracted. If wider range was accompanied by larger population size, as tends to be the case in animals (e.g. Blackburn *et al*., 2001), this would explain the relatively high diversity reported in *P. otagoense* (Goldberg *et al*., 2015). We examined this scenario using a combination of spatial, climatic, morphological and phylogeographic data to compare these species, and infer the processes shaping their range and variation. Ecological modelling of presence/absence data across the spatial and elevational range of New Zealand was used to confirm that the two species have distinct environmental envelopes and to infer the major variables associated with their spatial ranges. We sampled modern populations across the species' ranges and applied new Bayesian assignment tools to traditional morphometric traits to test the two-species hypothesis and classify individuals. With this sample, we then used mtDNA haplotype data to examine in more detail than previously matrilineal diversity and phylogeographic structure of the two species. mtDNA haplotype data together with allelic diversity at the rRNA internal transcribed spacer (ITS) locus, and geometric morphometric analysis of pronotum shape were used to examine whether range change had resulted in inter-specific introgression. Finally, we exam-ined the impact of rapid anthropogenic habitat modifica-tion by comparing evidence of potential and realised niche space.

Materials and methods

Grasshoppers

Phaulacridium is an Australasian genus of small short-horned grasshoppers (Acrididae) represented in southern Australia and New Zealand by endemic species. Their populations comprise one generation per year with egg diapause broken by cool winter temperatures, and peak adult activity in summer. They require open space for thermoregulation through basking and forage in natural and modified grasslands (Clark, 1967; Harper, 1972; Chapman, 1987; Scott, 1997), eating native herbs and small shrubs and exotic herbs including clover (*Trifolium repens*) and plantain (*Plantago lanceolata*). Native tussock grasses that dominate natural vegetation of open habitat in New Zealand are rarely eaten (Watson, 1970; and unpublished data). The commonest Australian species (*Phaulacridium vittatum*) is reported to have increased since European pasturalisation and is able to survive drought using deep rooted exotic weeds (Clark, 1967; Key, 1992), and although commonly called the wingless grasshopper *P. vittatum* is polymorphic for flight func-tional wings (Rentz, 1996). In New Zealand, fully winged *P. marginale* are occasionally recorded, but all *P. ota-goense* are micropterous. The two New Zealand species differ in shape, size (even when in sympatry), colour pat-tern and genome size (Westerman & Ritchie, 1984), and preliminary allozyme analysis indicates differences in allele frequencies (Scott, 1997). Where the two New Zealand species meet in Central Otago, *P. otagoense* is reported to favour the driest microhabitats while *P. marginale* uses lusher conditions as it does elsewhere in its range

(Westerman & Ritchie, 1984; Scott, 1997). Inter-species mating was reported by Westerman and Ritchie (1984), but they were unable to detect gene flow.

Environmental distinction of species ranges

To test whether the two New Zealand species of *Phaulacridium* have distinct environmental envelopes, we collated and mapped all available locality records, drawing on published material including Tenure Review reports (Appendix S1) prepared for the New Zealand Government's Department of Conservation, and our own observations. We used our database of field survey records spanning New Zealand from coastline to 2000 m above sea level, to generate a presence/absence matrix of New Zealand grasshoppers. This comprised 949 localities and records of 14 native New Zealand grasshopper species including 217 locations where *P. marginale* was present and 26 with *P. otagoense* (Fig. 1). This approach allowed inclusion of the large number of data points spanning potential range across New Zealand. Only mainland locations were used for mapping, despite the presence of *P. marginale* on some near-shore islands and the Chatham archipelago (Goldberg *et al.*, 2015).

In order to define and project the climatic envelopes of *P. marginale* and *P. otagoense*, the 19 current climate variables available from Worldclim (http://www.worldclim.org/) were downloaded at a resolution of 30 arc seconds (~1 km^2) (Appendix S2). Variables were cropped to the extent of New Zealand (Latitude 49°–32°S; Longitude 165°–180°E) using QGIS v2.16.1 (QGIS Development Team, 2017). We used variance inflation factor (VIF) analysis to refine the variable list to increase parsimony and minimise over-fitting during the modelling process. The R package 'VIF' (Lin *et al.*, 2015) implements stepwise selection to identify and remove collinear variables using VIF regression (Lin *et al.*, 2011). This reduced the informative and independent variables to seven. As it has previously been reported that soil and vegetation types influence ranges of some orthoptera (e.g. Nattier *et al.*, 2013; Weiss *et al.*, 2013), we added soil and vegetation data layers to our analysis. These were rasterised and scaled from their original files 'Fundamental Soil Layers New Zealand Soil Classification' and 'Vegetation Cover Map of New Zealand' obtained through the Landcare Research New Zealand Limited (https://www.landcareresearch.co.nz/resources/data/lris) Land Resource Information System (LRIS) portal (https://lris.scinfo.org.nz/). This brought the total number of predictor variables used in our analysis to nine.

We used the R package 'biomod2' v3.3-7 to individually model the environmental envelopes of the two focal grasshopper species (Thuiller *et al.*, 2016). Ten different modelling methods analysed the presence/absence data against the predictor variables: generalised linear model (GLM), generalised boosting model/boosted regression tree (GBM), generalised additive model (GAM), classification tree analysis, artificial neural network, surface range envelope (SRE), flexible discriminant analysis (FDA), multiple adaptive regression splines, random forest (RF) and maximum entropy (MAXENT). Descriptions of each model and their use within 'biomod2' can be found in Thuiller *et al.* (2009). All modelling parameters were kept at default values and 80% of the data were used to calibrate the models, with the remaining 20% being used to test them. Current climatic conditions were analysed using the nine variables, with each model repeated three times resulting in 30 environmental niche models. Importance values for each variable were calculated by subtracting the

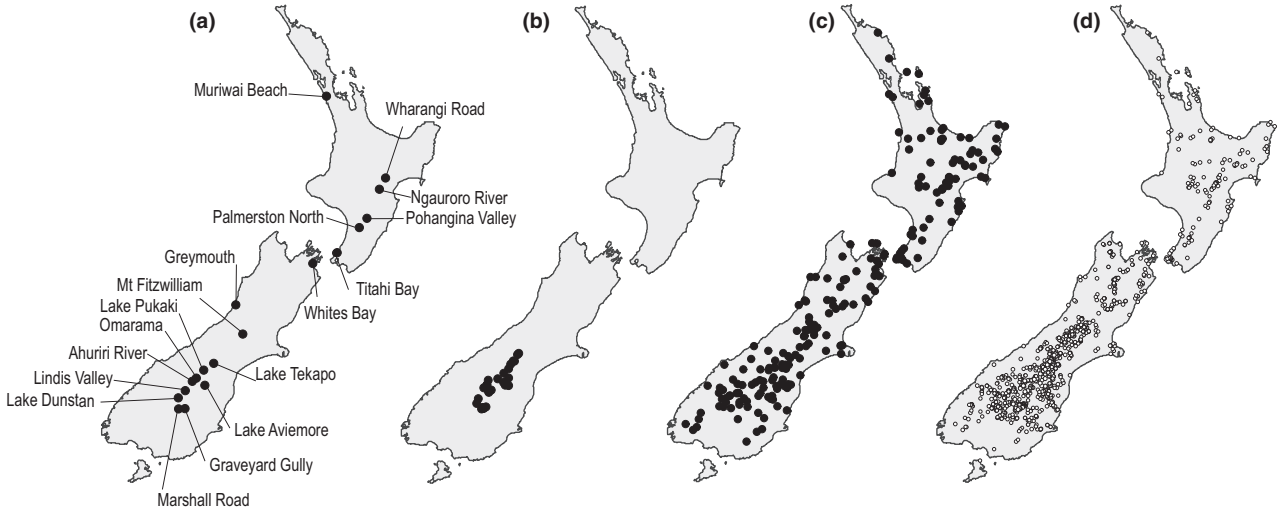

Fig. 1. *Phaulacridium* grasshoppers in New Zealand. (a) Source locations of specimens used for morphological and genetic analyses. (b–d) Spatial occurrence data for 16 species of New Zealand grasshoppers at 949 locations used in environmental modelling: (b) presence of *Phaulacridium otagoense* $n = 26$, (c) presence of *Phaulacridium marginale* $n = 217$, and (d) presence of other native grasshopper species predominantly from alpine zones (*Brachaspis*, *Sigaus*, *Paprides*, *Alpinacris*). All sites were searched for all taxa.

mean correlation score of each variable from 1, with scores closest to 1 indicating a variable of most importance in the model.

Environmental niche model accuracy was investigated using two different evaluation methods: receiver operating characteristic (ROC) and true skill statistic (Appendix S3). An ensemble model was then created from a subset of these models included if their ROC values were ≥0.9. Spatial plots of potential habitat distribution were produced using the ensemble mean weights model (EMmw). EMmw variable importance was calculated by applying the weights produced in the ensemble model to the associated models in the 30 model data set. Three runs of each model method were averaged, and the EMmw calculated by summing the total of these averages for each predictor variable and dividing by the number of modelling methods used (10). Final scores of variable importance were converted into percentages of total variable importance for each modelling method.

Past and future potential ranges of species were inferred using bioclimatic variables from the MIROC-ESM global climate models (Watanabe *et al*., 2011) for the LGM and 2070. These were at a resolution of 2.5 arc minutes (~5 km^2) for the LGM and 30 arc seconds (~1 km^2) for 2070. The future predicted climate layers RCP2.6 and RCP8.5 were used separately for the 2070 data set. These are two of the AR5 greenhouse gas concentration trajectories used by the inter-governmental panel on climate change (IPCC) and project an average global temperature increase of 1.0 and 3.7 °C respectively (IPCC, 2014). Using the ensemble model, species' potential range forecasts were projected for the LGM and the 2070 climate data. The soil and vegetation predictor variables were kept as static layers throughout the analysis as no past or future projections of such data are currently available for New Zealand. Models combining both static and dynamic predictor variables are known to perform as well as, or better than, models where only dynamic variables are included (Stanton *et al*., 2012). We ran all our analyses with and without the inclusion of data on soil and vegetation.

We also independently mapped natural and modified vegetation patterns of New Zealand as these likely represent important biological constraints on the spatial range of native grasshoppers (realised niche). To do this, we generated vegetation maps representing current and pre-human patterns using the 'Potential Vegetation of New Zealand' and 'Vegetative Cover Map of New Zealand' files obtained from the LRIS portal (https://lris.scinfo.org.nz/) (Newsome, 1987; Leathwick *et al*., 2012). The Potential Vegetation initiative seeks to provide a model of the distribution of natural vegetation types in New Zealand that would inform conservation and restoration projects and extends previous statistical modelling (Leathwick, 2001) to reconstruct vegetation pattern expected in the absence of human activity (Leathwick *et al*., 2012). Environmental data layers were drawn from the most recent iteration of the Land Environments of New Zealand classification (Leathwick *et al*., 2003) allowing grid resolution of 100 m. During modelling of these layers, particular attention was given to defining the treeline (Leathwick *et al*., 2012), which marks the boundary between forest and subalpine vegetation (notably *Chionochloa* tussock communities), and is an influential natural biological limitation for New Zealand grasshoppers (Bigelow, 1967). The files were edited in QGIS where broad vegetation classifications were regrouped as: indigenous forest; agricultural land; Tussock grassland/subalpine scrub; urban; wetland communities; sand-dune communities and unclassified categories.

Taxonomic identification with morphology

We collected 149 *Phaulacridium* grasshoppers from lowland grassland habitat at 18 locations by hand or sweep net (Fig. 1, Appendix S4). Nine locations were sampled in southern South Island New Zealand where both *Phaulacridium* species have been reported (Westerman & Ritchie, 1984; Key, 1992; Goldberg *et al*., 2015). We identified this sample of grasshoppers using data collected from adults for the diagnostic characteristics previously reported (Westerman & Ritchie, 1984). Adults were distinguished from juveniles by the presence of tegmina entirely covering the vestigial wings. Females were distinguished from males by the presence of an ovipositor. Eight non-independent variables were measured, comprising three size differences (hind femur length, hind tibia length and tegmina length), three ratios (hind femur length/width, pronotum width 3/pronotum length and pronotum width 1/pronotum width 2) and two angles of the pronotum margin in dorsal view (angle 1–PA1 and angle 2–PA2) (Appendix S5). Measurements were made using an Olympus SZX7 stereomicroscope with Olympus SC100 image capture and Olympus cellSens Dimension v1.6 software (Olympus Corporation, Tokyo, Japan).

In order to test the hypothesis of two morphologically distinct species of *Phaulacridium* in our sampling and assess signal in the data, we implemented a parameterised Gaussian hierarchical clustering algorithm to model the most likely number of clusters within the data and assign each specimen to the groups inferred. Model-based clustering used the Mclust package (Fraley & Raftery, 2003) in the R programming environment (R Development Core Team 2014). The Mclust v5.0.2 algorithm (Fraley *et al*., 2012) is built from a general model where the total data set is considered as a mixture of multivariate normal data sets, with a selection of covariance structures and vectors of expectation (Nanova, 2014). Unlike discriminant analysis, Mclust analysis does not require prior information about specimen identity to classify sample data. The optimal model of variance and number of clusters in the data are selected based on Bayesian information criteria (BIC), using the value of the maximised log likelihood with a penalty for the number of parameters in the model (Fraley & Raftery, 2003; Cordeiro-Estrela *et al*., 2008; Nanova, 2014). The higher the BIC score, the lower the

global average and median classification uncertainty, the better the model fits the data set (Cordeiro-Estrela *et al*., 2008). Additionally, under each model, the assignment probability of an individual belonging to a cluster can be computed (Fraley & Raftery, 2003).

Model-based clustering applied in this way allows exploration of the data, so we reran analyses varying the number and combination of variables included in order to optimise diagnostic signal. In particular, we sought to minimise non-independence of variables in analyses by examining signal resulting from use of one variable of each type (a linear dimension, a ratio and an angle). This revealed that three variables (hind femur length, hind femur length/width and pronotum angle 2) used in combination were sufficient to identify specimens to species. Additional variables did not alter results. Males and females were best treated separately as the grasshoppers are sexually dimorphic, primarily in size (females larger).

Demographic history of mtDNA lineages

Whole genomic DNA was extracted from foreleg tissue of recently collected or alcohol preserved specimens using a salting-out method (Sunnucks & Hales, 1996; Trewick & Morgan-Richards, 2005). Polymerase chain reaction (PCR) primers L2-N-3014 (Simon *et al*., 1994) and hopp-1490 (5′ TTTCAACAAACCATAAGGACATTGG 3′), modified from LCO1490 (Folmer *et al*., 1994), were used to target a 755 bp fragment of the mitochondrial DNA gene cytochrome oxidase subunit I (COI). PCR was performed in 20 μl volumes containing 0.2 μl of MCLAB Taq (Molecular Cloning Laboratories, South San Fransisco, CA, USA), 2.0 μl of 10× MCLAB buffer (Molecular Cloning Laboratories), 2.0 μl of 2 mM dNTPs, 1.4 μl of 25 mM Mg^{2+}, 0.8 μl of each primer (1 mM) and 2.0 μl of genomic DNA (~5 ng/μl). Thermocycling conditions were 95°C for 90 s; 94°C for 15 s, 52°C for 15 s and 72°C for 90 s repeated 38 times. Amplification products were checked on 1% TAE agarose gels and sequenced using BigDye v3.1 chemistry and an ABI3730 DNA analyzer. Sequences were edited and aligned using Geneious v9.1.4 (Kearse *et al*., 2012).

A Bayesian phylogenetic analysis using an outgroup of homologous sequence data from the endemic New Zealand *Sigaus australis* (Bigelow, 1967) and representatives of four species of Australian *Phaulacridium*, was used to establish statistical support for distinct clades within New Zealand *Phaulacridium*. A neighbour-joining tree, inferred with the Geneious tree builder, was used to examine the distribution of haplotypic variation within New Zealand *Phaulacridium*. Haplotype diversity (*h*), nucleotide site diversity (π, Nei, 1987) and the average number of nucleotide differences (*k*) within each *Phaulacridium* clade were calculated in DnaSP v5.10 (Librado & Rozas, 2009).

We examined the demographic history of mitochondrial lineages to infer past range changes of this non-recombining, unitary genome. We used mismatch distribution, the frequency distribution of pairwise mutational differences among individuals (Rogers & Harpending, 1992), to explore demographic signature in *Phaulacridium* genetic lineages with DnaSP v5.10 (Librado & Rozas, 2009). The distribution of pairwise differences in stable populations is expected to be multimodal compared to a unimodal distribution for populations that have undergone recent demographic expansion (Rogers & Harpending, 1992; Harpending, 1994). Goodness of fit to a unimodal mismatch distribution was tested using the Harpending's raggedness index (Harpending *et al*., 1993) and the sum of squares deviation (SSD; Schneider & Excoffier, 1999) using Arlequin v3.5 (Excoffier & Lischer, 2010) with 1000 bootstrap resamples. A non-significant result indicates a good fit and support for population expansion.

In addition, three neutrality tests, Tajima's *D* (Tajima, 1989), Fu's F_S (Fu, 1997), and Ramos-Onsins and Rozas's R_2 (Ramos-Onsins & Rozas, 2002), implemented in DnaSP v5.10 (Librado & Rozas, 2009), were used to detect departures from the mutation–drift equilibrium that would be indicative of changes in historical demography and natural selection. We assessed statistical significance with 1000 permutations. Values near zero for Tajima's *D* and Fu's F_S imply constant population size. Significant negative values imply population expansion or purifying selection, while positive values indicate population subdivision or recent population bottleneck. Ramos-Onsins and Rozas's R_2 and Fu's F_S are considered the best statistical tests for sensing population growth, with R_2 being better suited to small sample sizes than F_S (Ramos-Onsins & Rozas, 2002). Significant R_2 values close to zero are interpreted as implying population expansion.

Evidence of introgression

Pronotum shape. We sought evidence of introgression between the two *Phaulacridium* species by examining the match between mtDNA lineage and specimen identification. In addition, we used geometric morphometrics to analyse fine-scale shape variation, as inter-species gene flow would be expected to yield individuals with a mixed morphological phenotype (e.g. Gómez *et al*., 2013; Pentinsaari *et al*., 2014). We used pronotum shape as this structure is not susceptible to arbitrary changes during preservation (cf. whole body dimensions), and has previously been shown to be amenable to the methods used (e.g. grasshoppers–Dowle *et al*., 2014; beetles–Ober & Connolloy, 2015). The pronotum of *Phaulacridium* is known to have features that differ between the described species, but this has never been formally analysed in terms of shape. Geometric analysis allows elimination of isometric size variation by superimposition via Procrustes transformation, while capturing all shape variability of the raw landmark data (Bookstein, 1991; Rohlf & Marcus, 1993; Klingenberg, 2016). We included only adults in our study, thereby avoiding allometric effects on shape associated with ontogeny. We obtained data from each grasshopper using twelve landmarks on the perimeter of the dorsal

surface of the pronotum using digital images on a Wacom Cintiq 22HD digitising tablet and tspDIG v2.17 software (Rohlf, 2013) (Appendix S5). High-resolution digital images were captured with an Olympus SC100 and Olympus SZX7 stereomicroscope using Olympus cellSens Dimension v1.6 software. The landmarks were selected for their ease of identification, homology among grasshopper individuals, and their capacity to capture the major shape features of the structure.

Measurement error was minimised by careful mounting, photography and landmark selection, and measurement error associated with photographing and digitising the images was assessed by reimaging and digitising one grasshopper 10 times. A Procrustes analysis of variance (Procrustes ANOVA) was used to compare variation in landmark location among iterations. The scale of the measurement error was assessed by comparison of the mean squares of pronotum shape variation and confirmed to be significantly lower than biological variation.

A Procrustes fit aligned by principal axes was performed to eliminate size differences, and linearly uncorrelated variables were obtained from a principal component analysis (PCA) across all individuals and all landmarks in MORPHOJ (Klingenberg, 2011). We analysed the principal components accounting for most variation, using the model-based clustering approach of Mclust v5.0.2 (Fraley *et al*., 2012) to identify the optimal number of clusters of shape variation in the data, and their composition.

Nuclear rRNA diversity. Allelic variation at a nuclear locus would confirm gene flow; however, single copy neutral nuclear loci have not been developed for these species. Instead, preliminary evidence of introgression at nuclear loci was sought by comparing 45S ribosomal cassette DNA sequences from three representative specimens using a genomic approach. Eukaryote genomes contain numerous copies of the 45S rRNA cassette, but minimal variation among copies is normally maintained by concerted evolution (Ganley & Kobayashi, 2007). High copy number makes the 45S locus amenable to next generation sequencing (NGS) approaches, while concerted evolution means any allelic variation encountered is most likely to be the result of recent introgression (e.g. Trewick, 2001; Trewick *et al*., 2008; Wan *et al*., 2014). Total genomic DNAs from three individuals were separately processed through massive parallel, high-throughput sequencing (Illumina HiSeq 2500, Illumina, San Diego, CA, USA) for a separate phylogenetic study. Genomic DNA was fragmented, prepared using the ThruPLEX® DNA-seq Kit (Rubicon Genomics, Ann Arbor, MI, USA) and used to generate 100 bp paired-end sequence. The scale of sequencing did not provide sufficient data to assemble single copy nuclear genes as Acrididae have large genomes approximately three times bigger than humans (e.g. the related grasshopper *P. vittatum* has a haploid genome of 10.73 pg); however, it was sufficient for rich coverage of multicopy nuclear markers including the 45S rRNA cassette.

Adapter sequence barcodes and poor-quality data were removed from IIlumina sequence reads using cutadapt (Martin, 2011). Geneious v9.1.4 (Kearse *et al*., 2012), was used to pair and map sequence reads and to align resulting consensus sequences. The entire 45S was initially assembled by aligning paired reads to an annotated reference sequence from the New Zealand grasshopper *Paprides nitidus* (unpublished) that belongs to the same subfamily (Cantanopinae). The resulting consensus sequence was used as a reference for iterative remapping of raw read data until gaps were filled by extension with new sequence data. The highly conserved 5.8S locus within 45S was then excised and used for separate, iterative mapping of each set of raw sequence reads in order to assemble the flanking ITS 1 and 2 alleles. Sequence variants within the ITS region (including 5.8S, ITS1 and 2) were identified for each individual by identifying consistent single nucleotide substitutions among the paired reads when compared to the reference sequence. An unrooted network of sequence variants for the three New Zealand *Phaulacridium* was constructed using the median joining algorithm (Bandelt *et al*., 1999) in PopART (Leigh & Bryant, 2015).

Results

Environmental envelopes

A total of 243 locations for *Phaulacridium* were obtained from our own observations and published records dating from 1959 to the present, and mapped (Fig. 1). *Phaulacridium marginale* was recorded throughout New Zealand and many surrounding islands. In contrast, the distribution of *P. otagoense* was limited to the central/southern South Island.

Analyses with and without the inclusion of soil and vegetation data produced similar results, implying that climate data provide a proxy for the key attributes dictating grasshopper distribution. Natural vegetation at the scale relevant to these grasshoppers (i.e. open grassland/scrub vs. evergreen forest) is primarily a function of climate. Most environmental models for *P. marginale* found annual mean temperature to have the highest predictive power of the seven climate variables included (Table 1). The ensemble model combined models using four methods (GLM, GBM, GAM, RF, all with ROCs > 0.9) resulting in annual mean temperature (60%), and mean diurnal range (14.59%) the most important variables in predicting where *P. marginale* occurs. Environmental models for *P. otagoense* differed in finding mean diurnal temperature range to be most influential (Table 2). The ensemble model for this species combined models using four methods (GLM, GBM, FDA, MAXENT).

The potential distribution of *P. marginale* and *P. otagoense* can be inferred for the past and future climate of New Zealand (Fig. 2). For *P. marginale*, suitable

Table 1. Predictive power of climate variables in determining the range of the New Zealand grasshopper *Phaulacridium marginale*, using ten modelling methods* and combining the best fitting models (EMmw).

Average%	GLM	GBM	GAM	CTA	ANN	SRE	FDA	MARS	RF	MAXENT	EMmw
Annual mean temperature	67.53	66.76	78.53	78.59	32.28	22.67	73.17	72.44	38.02	71.08	60.11
Mean diurnal temperature range	12.96	10.72	6.39	17.09	5.55	9.03	5.93	13.28	7.62	3.51	9.21
Isothermality	3.42	0.76	1.09	0.00	0.87	18.53	3.08	0.60	2.67	1.14	3.22
Mean temp of wettest quarter	0.00	10.60	0.00	2.10	13.00	24.98	6.25	0.60	13.79	5.26	7.66
Mean temp of driest quarter	9.60	4.20	2.09	0.00	12.30	5.83	0.05	5.38	20.14	13.76	7.34
Precipitation of driest month	0.00	2.28	3.48	0.00	9.97	8.29	10.78	5.25	5.11	0.82	4.60
Precipitation seasonality	4.12	1.69	1.51	0.00	3.07	7.95	0.66	1.49	1.74	1.16	2.34
Soil type	0.62	0.47	2.54	0.00	9.86	1.62	0.00	0.00	1.45	0.24	1.68
Vegetation type	1.76	2.52	4.37	2.22	13.10	1.08	0.08	0.97	9.46	3.03	3.86

Predictive power of each variable under each model was given by the correlation between the post-training standard score and the score from a new prediction made after randomising the given variable. Low correlation indicates strong influence of the variable on the model. Here, predictive power of the variable is proportionated for each model separately (as a percentage); a high value indicates high importance of that variable in the model. The most influential variable for each model is indicated with grey background.
*Model acronyms are as follows: GLM, generalized linear model; GBM, generalised boosting model/boosted regression tree; GAM, generalised additive model; CTA, classification tree analysis; ANN, artificial neural network; SRE, surface range envelope; FDA, flexible discriminant analysis; MARS, multiple adaptive regression splines; RF, random forest; MAXENT, maximum entropy; EMmw, ensemble model mean weights model.

Table 2. Predictive power of climate variables in determining the range of the New Zealand grasshopper *Phaulacridium otagoense*, using ten modelling methods and combining the best fitting models (EMmw).

Average%	GLM	GBM	GAM	CTA	ANN	SRE	FDA	MARS	RF	MAXENT	EMmw
Annual mean temperature	22.36	3.17	0.00	39.66	12.09	12.64	0.00	0.72	6.43	0.00	9.71
Mean diurnal temperature range	50.04	51.15	64.36	60.34	16.37	23.99	72.41	40.26	35.37	63.61	47.79
Isothermality	12.49	0.08	0.00	0.00	3.21	9.64	0.00	3.36	0.38	3.81	3.30
Mean temp of wettest quarter	1.11	0.41	0.00	0.00	8.47	7.52	11.59	5.21	13.34	0.00	4.77
Mean temp of driest quarter	1.52	23.16	0.00	0.00	15.33	13.04	1.41	3.85	5.77	3.70	6.78
Precipitation of driest month	4.41	5.66	0.00	0.00	23.14	13.00	0.00	12.30	17.76	12.72	8.90
Precipitation seasonality	0.94	14.56	0.40	0.00	5.26	9.94	0.00	19.60	9.38	9.05	6.91
Soil type	2.63	0.87	1.44	0.00	7.42	6.61	12.24	3.56	1.67	1.18	3.76
Vegetation type	4.50	0.95	33.80	0.00	8.71	3.61	2.35	11.13	9.90	5.94	8.09

The most influential variable for each model is indicated with grey background (see Table 1 for further details).

climatic conditions were widespread during the LGM with distribution likely in most of northern and western New Zealand. Suitability of southern New Zealand increased to the present and is predicted to further increase in the future (2070 projection), as models with warming of 2.2 °C would increase climatically suitable areas for *P. marginale* (Fig. 2). Climatically suitable habitat for *P. otagoense* during the LGM was inferred as little different from that predicted for current conditions in Central Otago, South Island, that has the lowest moisture levels in New Zealand (Leathwick *et al*., 2003). In contrast, cold dry winters similar to those currently experienced in Central Otago were also indicated for northeast New Zealand where the species is not recorded. Projections of future climate, suggest some increase in suitable area for *P. otagoense* based on our ensemble environmental model (Fig. 2), and this is consistent with the prediction that eastern dry areas will tend to get drier (Harrington & Renwick, 2014; Renwick *et al*., 2016). The finding that the region of South Island in which *P. otagoense* occurs today has conditions that are mostly the least suitable for *P. marginale*, confirms that the two taxa are ecologically different.

Taxonomic identification and range changes in 30 years

We found three morphological traits reported to be diagnostic for the two grasshopper species (hind femur length, hind femur length/width and pronotum angle 2) (Westerman & Ritchie, 1984) were effective for distinguishing taxa. Bayesian assignment found support for two morphological clusters for each sex (Appendices S6, S7), consistent with our expectations of two *Phaulacridium* species. The model with best statistical support for male *Phaulacridium* (BIC = −537.92) was EEI (diagonal, equal volume and shape) and for female *Phaulacridium* (BIC = −744.65) was EVI (diagonal, equal volume, varying shape). A total of 124 (56 males, 68 females) grasshoppers were determined to be *P. marginale*, and 24

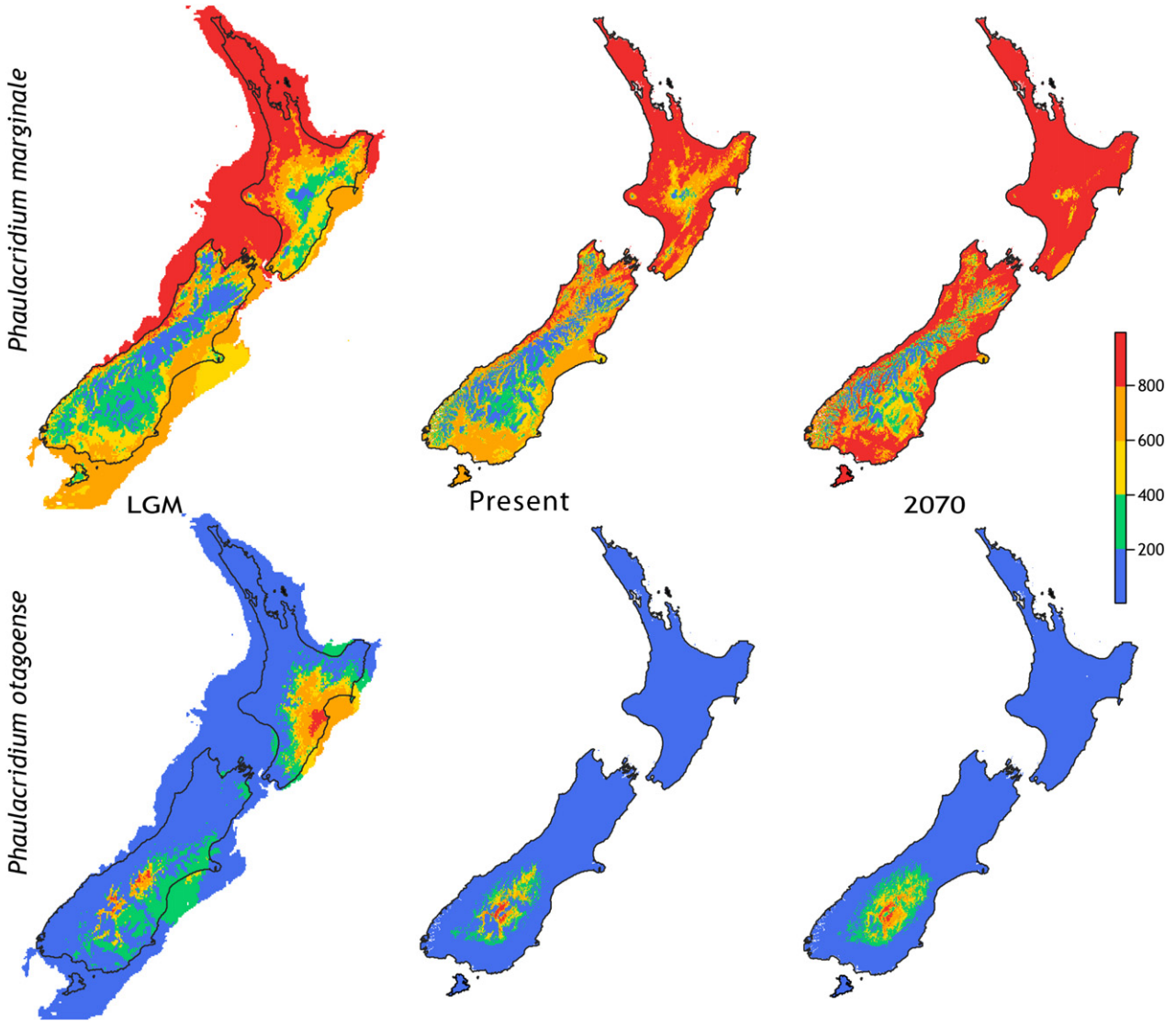

Fig. 2. Contrasting climatically suitable regions of New Zealand inferred for two lowland *Phaulacridium* grasshopper species: Upper row, widespread *P. marginale*; Lower row, restricted *P. otagoense*. The ensemble model mean weights combined the best fitting models from scenarios based on seven climate variable plus vegetation and soil data. Range probabilities were estimated for last glacial maxima; present; and future (2070; based on predicted global warming of 2.2 °C). Warm colours (e.g. red) equate to high probability of suitable climate, cool colours (e.g. blue) indicate low probability of suitable climate. GLM, generalised linear model.

(7 males, 17 females) were *P. otagoense* (Appendix S4). Most (94.6% of the sample) assignment probabilities of individuals to a species were ≥0.9. The northern South Island locations and all of the North Island populations contained only *P. marginale*. *Phaulacridium otagoense* was collected from five southern South Island locations, but at three of these we also collected *P. marginale* (Table 3). We found only *P. marginale* at Omarama and Lake Aviemore, both places where *P. otagoense* has previously been collected (Westerman, 1974, 1983; Westerman & Ritchie, 1984), and we found both species at Graveyard Gully that was formerly occupied only by *P. otagoense*.

Demographic history of mtDNA lineages

The 755 bp alignment of homologous COI sequences from 149 *Phaulacridium* individuals contained 52 unique mtDNA haplotypes, with 99 of these base pair positions being variable (13%) and 71 (9%) being parsimony informative sites. Phylogenetic analysis of the haplotypes revealed four lineages (clades) rather than two as expected from sampling two species (Fig. 3, Appendix S8). Distinct clades were confirmed by posterior probabilities of 1.0 from Bayesian phylogenetic analysis of DNA sequences with an outgroup. Lineage I was equivalent to the previously reported

Table 3. Population samples of *Phaulacridium* grasshoppers in region of sympatry (central South Island New Zealand) display some mismatch between morphology and mtDNA.

			Pronotum		mtDNA	
Specimen code	Location	Species	Cluster	*P*	Lineage	Haplo.
GH1464	Lake Tekapo	***otagoense***	**C**	**1.00**	**IV**	**45**
GH1465		juvenile	–		I	29
GH1466		*otagoense*	C	1.00	I	23
GH1467		*otagoense*	C	0.99	I	23
GH1468		*otagoense*	B	0.94	I	29
GH1469		*otagoense*	C	0.99	I	29
GH1474	Lake Pukaki	***marginale***	**A**	**0.64**	**I**	**28**
GH1475		*marginale*	B	0.95	IV	44
GH1476		*otagoense*	A	0.86	I	28
GH1477		*marginale*	C	0.87	IV	43
GH1479		***marginale***	**A**	**0.84**	**I**	**23**
GH1480		***marginale***	**A**	**0.76**	**I**	**29**
GH1484		*marginale*	C	0.93	I	29
GH1485		***marginale***	**A**	**0.95**	**I**	**1**
GH1494		***marginale***	**B**	**0.94**	**I**	**23**
GH1499	Lake Aviemor	*marginale*	A	1.00	IV	52
GH1500		***marginale***	**A**	**0.99**	**I**	**25**
GH1501		***marginale***	**A**	**1.00**	**I**	**23**
GH1502		***marginale***	**A**	**0.98**	**I**	**29**
GH1503		***marginale***	**A**	**0.98**	**I**	**29**
GH1504		*marginale*	B	0.95	IV	51
GH1506		*marginale*	B	0.89	IV	52
GH1507		***marginale***	**B**	**0.91**	**I**	**29**
GH1512		***marginale***	**B**	**0.98**	**I**	**28**
GH1513		***marginale***	**B**	**0.93**	**I**	**29**
GH1519	Omarama	*marginale*	C	0.87	I	29
GH1522		***marginale***	**A**	**1.00**	**I**	**29**
GH1524		*marginale*	A	0.99	IV	48
GH1525		*marginale*	B	0.97	IV	49
GH1526		***marginale***	**A**	**0.91**	**I**	**29**
GH1527		*marginale*	A	0.97	IV	47
GH1529		***marginale***	**B**	**0.62**	**I**	**29**
GH1532		***marginale***	**B**	**0.94**	**I**	**28**
GH1536	Ahuriri	***marginale***	**A**	**1.00**	**I**	**4**
GH1538	River	***marginale***	**A**	**0.93**	**I**	**19**
GH1539		*marginale*	A	0.67	IV	50
GH1540		*marginale*	C	0.73	IV	46
GH1541		***marginale***	**B**	**0.72**	**I**	**29**
GH1542		***marginale***	**B**	**0.98**	**I**	**29**
GH1543		***marginale***	**B**	**0.98**	**I**	**29**
GH1544		***marginale***	**B**	**0.98**	**I**	**29**
GH1548		***marginale***	**A**	**1.00**	**I**	**4**
GH1553		***marginale***	**B**	**0.67**	**I**	**18**
GH1556	Lindis	*otagoense*	C	1.00	I	29
GH1557	Valley	***otagoense***	**C**	**1.00**	**II**	**36**
GH1559		***otagoense***	**C**	**0.99**	**II**	**35**
GH1560		***otagoense***	**C**	**1.00**	**II**	**37**
GH1561		*otagoense*	C	0.99	I	29
GH1562		*otagoense*	C	1.00	I	29
GH1563		***otagoense***	**C**	**1.00**	**II**	**34**
GH1566		***otagoense***	**C**	**1.00**	**II**	**38**
GH1572		***otagoense***	**C**	**1.00**	**II**	**37**

(continued)

Table 3. (continued)

Specimen code	Location	Species	Pronotum		mtDNA	
			Cluster	*P*	Lineage	Haplo.
GH1574	Lake Dunstan	*marginale*	C	0.73	II	32
GH1575		***otagoense***	**C**	**1.00**	**II**	**33**
GH1576		***otagoense***	**C**	**1.00**	**II**	**30**
GH1578		***otagoense***	**C**	**1.00**	**II**	**31**
GH1580	Graveyard Gully	*otagoense*	B	0.58	III	41
GH1581		***marginale***	**B**	**0.88**	**I**	**28**
GH1582		***marginale***	**B**	**0.82**	**I**	**29**
GH1583		***marginale***	**A**	**1.00**	**I**	**28**
GH1584		*otagoense*	C	1.00	I	28
GH1585		*otagoense*	C	0.98	I	28
GH1586		*otagoense*	A	0.63	III	42
GH1588		*otagoense*	C	1.00	I	28
GH1589		*otagoense*	C	1.00	I	29
GH1599	Marshall Road	***marginale***	**A**	**0.98**	**I**	**29**
GH1600		*marginale*	B	0.97	III	39
GH1601		***marginale***	**A**	**0.99**	**I**	**29**
GH1602		*marginale*	A	0.99	III	40
GH1604		***marginale***	**A**	**0.99**	**I**	**29**
GH1605		***marginale***	**B**	**0.98**	**I**	**29**
GH1606		***marginale***	**B**	**0.97**	**I**	**29**
GH1607		***marginale***	**B**	**0.96**	**I**	**23**

Individual grasshopper codes and collection location of nine population samples are shown with morphological and genetic information. Classification to species used three traditional morphological traits (and model-based clustering). Bayesian assignment of pronotum shape (PC1 and PC2 from landmark geometric morphometrics) revealed three clusters in the optimal model (A, B, C; $K = 3$). Grasshopper specimens with no evidence of hybridisation are in bold. mtDNA cytochrome oxidase subunit I sequences for every individual were grouped by lineage and haplotype code (Haplo.). Location names are as given in Fig. 1.

P. marginale clade, whereas our lineages III and IV correspond to the two clades associated with *P. otagoense* (Goldberg *et al*., 2015). Lineage II had not been encountered before.

All four the haplotype lineages were found in both grasshopper species (Table 3), but haplotype diversity was spatially structured. Lineage I haplotypes were found in all population samples except Lake Dunstan in the south (Fig. 3). In contrast, lineages II, III and IV haplotypes were found only in grasshoppers from within the southern/central South Island range of *P. otagoense* (Fig. 3). Lineages III and IV were restricted to individuals from Alexandra and the Mackenzie Basin respectively. Lineage II was restricted to grasshoppers from Lindis Valley and Lake Dunstan (Otago) and haplotypes from these locations formed separate subclades (Fig. 3).

Because capture of divergent haplotypes will influence estimates of genetic diversity we analysed genetic variation after coding our data in each of three ways: (i) mtDNA haplotype diversity coded by two species (using grasshopper morphology); (ii) each of four mtDNA haplotype lineages analysed separately; and (iii) mtDNA haplotype diversity organised as geospatial groups, recognising and combining three haplotype lineages unique to the region of *P. otagoense* (II, III and IV) and lineage I representing *P. marginale* (this might represent intra-specific diversity before hybridisation). Although the sample size of *P. marginale* was much greater ($n = 124$, 41 haplotypes) than *P. otagoense* ($n = 24$, 14 haplotypes), both nucleotide site diversity (π) and haplotype diversity (h) were higher for *P. otagoense* (Table 4). When each haplotype lineage was analysed separately, lineage IV ($n = 11$) had the highest genetic diversity ($\pi = 0.016$) and lineage I the lowest ($n = 124$; $\pi = 0.003$). Among the 124 *Phaulacridium* individuals in mtDNA haplotype lineage I, there were 29 unique haplotypes with an average of 2.2 nucleotide differences between them ($\pi = 0.003$; Table 4). In contrast, within lineages II, III and IV, 25 individuals had 23 haplotypes with an average of 22.1 nucleotide differences ($\pi = 0.029$; Table 4). Nucleotide diversity within haplotype lineages (Table 4) therefore contrasts with the current geographical area in which they occur. The low genetic diversity of the widespread lineage I could be the result of recent population expansion. In contrast, the diversity within the Otago lineages II, III and IV suggests a relatively large stable population.

We inferred demographic histories of the mitochondrial lineages to estimate past range changes of this maternally inherited genetic marker. Mismatch distribution for lineage I was unimodal and smooth (Fig. 3) consistent with recent demographic expansion. Demographic expansion of lineage I was further supported by a low and statistically insignificant Harpending's raggedness index (0.024, $P > 0.05$) and a low SSD value (0.002, $P < 0.05$). Tajima's D and Fu's F_S were significantly negative ($D = -1.963$, $P < 0.01$;

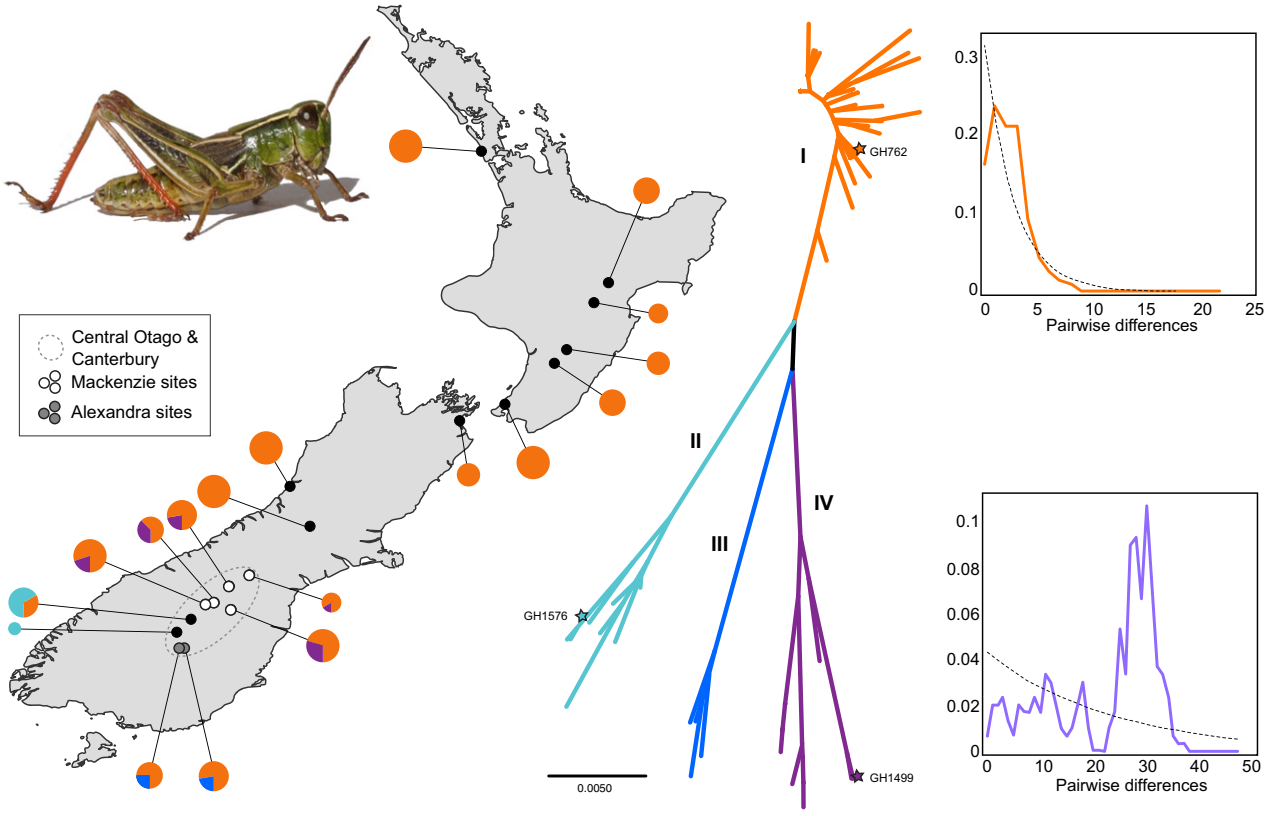

Fig. 3. Mitochondrial DNA cytochrome oxidase subunit I haplotype variation among New Zealand *Phaulacridium*. The relative frequency and spatial distribution among population samples (left) of the four mitochondrial lineages shown in the unrooted neighbour-joining tree (centre). The haplotypes of the three specimens that provided nuclear rRNA internal transcribed spacer sequence data are indicated by stars. Mismatch distribution plots for mtDNA lineage I (top right) and lineages II, III and IV (bottom right) show contrasting degrees of raggedness.

Table 4. Descriptive statistics for four mtDNA cytochrome oxidase subunit I sequence lineages observed in two species of New Zealand *Phaulacridium* grasshoppers.

Lineage	n	N_{haps}	k	π	S	h
P. otagoense	24	14	17.043	0.02257	67	0.917
P. marginale	124	41	6.845	0.00907	87	0.886
Lineage I	124	29	2.191	0.00290	34	0.841
Lineage II	10	9	7.489	0.00992	22	0.978
Lineage III	4	4	5.833	0.00773	11	1.000
Lineage IV	11	10	11.891	0.01575	28	0.982
Lineages II, III & IV	25	23	22.140	0.02932	74	0.993

Sample size (n), number of observed haplotypes (N_{haps}), average number of nucleotide differences (k), nucleotide diversity (π), number of polymorphic sites (S) and haplotype diversity (h). The total number of identified specimens was 148, whereas the total number of specimens sequenced was 149 as it included one juvenile grasshopper without species id.

$F_S = -21.109$, $P < 0.001$) for lineage I haplotypes, with Ramos-Onsins and Roza's R_2 being significantly low (0.032, $P < 0.05$). These results are indicative of population expansion of mitochondrial lineage I, although a selective sweep could produce a similar effect.

In contrast, by grouping haplotypes unique to the narrow range of *P. otagoense* (lineages II, III and IV) a more ragged and multimodal distribution is revealed that is typical of population size at demographic equilibrium (Fig. 3). The mismatch distribution goodness of fit tests were not significant (Harpending's raggedness index 0.121, $P > 0.05$; SSD = 0.013, $P > 0.05$), indicating that there was no severe departure from the estimated demographic model. For *P. otagoense* lineages, Tajima's D and Ramos-Onsins and Roza's R_2 were non-significant ($D = 0.176$, $P > 0.05$; $R_2 = 0.141$, $P > 0.05$), signifying population expansion had not occurred. In contrast, Fu's F_S was significantly negative (-5.623, $P < 0.05$), which could be a result of the low population size or low sample size (Ramos-Onsins & Rozas, 2002).

Detecting introgression

Pronotum shape. Hybridisation between the grasshopper species might lead to individuals with intermediate

phenotype, so we examined the shape of adult pronota in more detail. Geometric morphometric analysis of 12 pronotum landmarks found highly significant differences between pronotum shapes (MS pronotum = 0.004148, MS error = 0.000027, $P < 0.0001$) with Procrustes ANOVA. The mean squares for pronotum shape variation exceeded the mean squares for measurement error 157-fold, indicating measurement error would have a negligible influence on analysis of the landmark locations on the pronotum.

Data from males and females were combined as the isometric influence of size was removed through superimposition of the raw data. We analysed principal components of pronotum shape variation obtained from MORPHOJ (Klingenberg, 2011) using model-based clustering without priors. The first two principal components explained 75% of the observed morphological variance in the sample (PC1 = 47.81%, PC2 = 27.54%), and were sufficient to reveal three pronotum shape clusters within our sample using BIC in Mclust v5.0.2 [VEI model (diagonal, equal shape); BIC = 1105.19]. One pronotum shape cluster contained most (83%) of the *P. otagoense* grasshoppers in our sample (both sexes). The two other clusters corresponded to female and male *P. marginale*, showing that in this species sexes differ in shape as well as size (Fig. 4). Overall, *P. otagoense* had a wider pronotum than *P. marginale*, and had more acute external angles on the lateral carinae.

Nuclear rRNA–ITS diversity. To further test whether recent contact between species has resulted in genetic introgression, we examined allelic diversity at ITS using high-throughput sequencing of whole genomic DNA. This yielded 1.17×10^9–2.34×10^9 bp of sequence (<20% of the genome) from each of three grasshopper individuals. Of this between 9245 and 30 797 reads assembled to the 45S rDNA cassette. After annotation, we extracted alignments of the ITS regions and identified a total of five ITS sequence variants (830 bp consisting of ITS1-5.8S-ITS2). All three *Phaulacridium* specimens, each representing a different mtDNA haplotype lineage (Fig. 3), had more than one ITS sequence variant. The DNA alignment included a short (5 bp) insertion/deletion region, resulting in some of the difference between the five sequences and preventing resolution of sequence variation via Sanger sequencing of this marker. The *P. otagoense* specimen had four different ITS sequence variants (a, b, c, d; Appendix S9). In contrast, both *P. marginale* grasshoppers each had only two ITS sequences (d, e).

Introgression

The majority of grasshoppers we collected were *P. marginale* with pronotum shape characteristic of that species and mtDNA haplotypes from lineage I ($n = 59$). In contrast, many *P. marginale* had either a pronotum shape characteristic of *P. otagoense* and/or a haplotype from lineages II, III and IV ($n = 30$). Likewise, some *P. otagoense* specimens had concordant traits for that species ($n = 10$), but more had a pronotum shape or mtDNA haplotype characteristic of *P. marginale* ($n = 14$). Of the eight population samples with more than one haplotype lineage, five had specimens with mismatch of species and pronotum shape, as expected of hybrids. Where the species and the mtDNA haplotype did not concur, we inferred it likely that hybridisation had led to mtDNA introgression.

Geometric analysis of pronotum shape for individuals previously classified by Bayesian clustering of traditional morphological characters revealed that five *P. marginale* specimens in our sample had a pronotum shape more typical of *P. otagoense*, and four *P. otagoense* specimens had a pronotum shape nearer that of *P. marginale* (Table 3). Cluster assignment probabilities for each individual revealed that a minority of grasshoppers (28 of 148) were assigned to clusters with a probability <0.9. Most of these came from locations where both species had been collected (Fig. 4, Appendix S8). The rRNA cassette surveyed from NGS data revealed abnormally high ITS variation in the specimen of *P. otagoense*, most likely the result of recent introgression from *P. marginale*.

Discussion

The geographical range of a species has been regarded as one of the most significant factors influencing genetic diversity and its distribution (Endler, 1977). Generally, it has been accepted that species with restricted ranges exhibit lower genetic diversity than species that are more widespread. Although population density might vary among taxa, population size at equilibrium is positively correlated with genetic diversity (Charlesworth, 2009) and with range size (e.g. Blackburn *et al*., 2001). Neither previous surveys (Westerman & Ritchie, 1984) nor our observations suggest that *P. otagoense* exists in significantly denser populations than *P. marginale*, and both species have strictly annual lifecycles. Therefore, the smaller spatial range of *P. otagoense* suggests this species has a smaller population size, predicting lower genetic diversity, compared to the more widespread *P. marginale*, which is the reverse of the relative genetic diversity documented here. Similar patterns of contrasting genetic diversity have been observed in other organisms including rare insect and plant species with high diversity compared to widespread congeneric species; in these studies, recent human-mediated reduction in population size was inferred (e.g. Chen *et al*., 2007; Bàlint *et al*., 2012).

The observed relative level of genetic diversity in these two grasshopper species was a reversal of expectations based on current species' range size and can be explained by a temporal lag in population genetic structure (Charlesworth, 2009; Landguth *et al*., 2010). A temporal lag is supported by our demographic history analyses. We inferred recent population expansion of *P. marginale*, while *P. otagoense* has retained a signature of

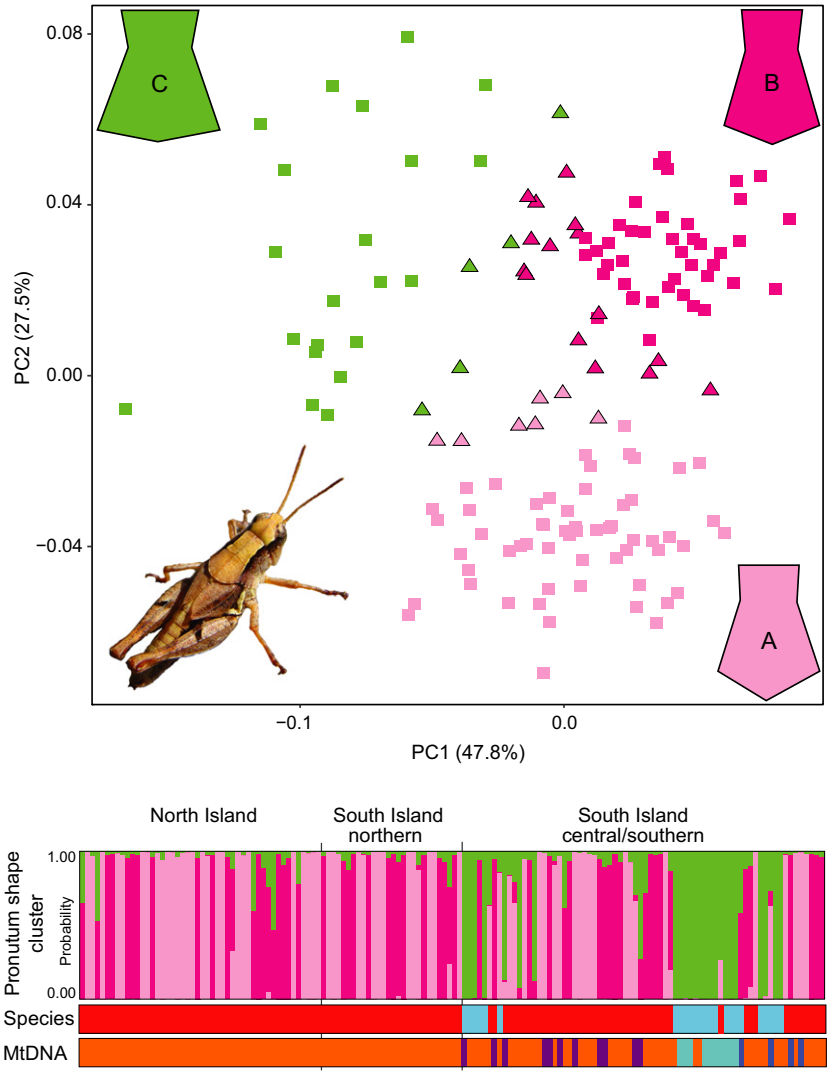

Fig. 4. Geometric morphometric analysis of pronotum shape reveals limited phenotypic introgression of New Zealand *Phaulacridium* grasshopper species. Top: Bayesian assignment of pronotum geometric landmark data (PC1 and PC2), without priors, found most support for a model with three phenotypic clusters. Symbols in the classification plot indicate pronotum shape of female *P. marginale* (cluster A, pink, $n = 65$) and male *P. marginale* (cluster B, magenta, $n = 57$), and *P. otagoense* (cluster C, green, $n = 26$). Squares indicate high assignment probability (≥0.9), and triangles show lower assignment probability (<0.9), corresponding to intermediate forms. Representative pronotum shapes of each cluster are shown. Bottom: Bar plot of assignment probabilities of each pronotum to the three model-based clusters, arranged by geographical region. Also shown are species assignments from traditional diagnostic characters (colours as in Appendix S7) (red = *P. marginale*; blue = *P. otagoense*), and mtDNA lineage (colours as in Fig. 3) of each grasshopper.

comparatively large, stable population history. Although high intra-specific haplotype diversity might also result from combination of lineages associated with cryptic taxa (Galtier *et al.*, 2009), our detailed morphological examination and spatial evidence support just one taxon spanning haplotype lineages II, III and IV. Contrary to the widely held idea that species should typically comprise single mitochondrial lineages (e.g. Tavares & Baker, 2008), simulation studies show that large stable populations are expected to have multimodal distributions because the history of coalescent events imposes a substantial correlation on this non-recombining DNA sequence (Slatkin & Hudson, 1991). Similar intra-specific diversity of mtDNA has been demonstrated in other Orthoptera (Morgan-Richards *et al.*, 2017).

Species range and environmental envelope

Contrasting population sizes in the recent past might be the result of *P. otagoense* reduction and/or *P. marginale* increase. Natural climate cycling through the Pleistocene

provides a prediction of changing fortunes in related species with different environmental envelopes (Parmesan, 2006; Stewart *et al*., 2010). This is in line with phylogeographic evidence of range change linked to climate shifts in the Northern Hemisphere (Hewitt, 2004). Available information for New Zealand (e.g. Newnham *et al*., 2013) predicts retraction of forest and expansion of grasslands associated with cooler, drier conditions during the LGM. Perhaps, *P. otagoense* had a bigger range and thus larger population during the LGM (Vandergoes *et al*., 2013; Williams *et al*., 2015)?

We investigated the possibility that species' range differences related to natural climate change since the LGM using ecological niche modelling. We found that although the two *Phaulacridium* species in New Zealand have contrasting distributions leading to distinct models of their environmental envelopes, climate modelling did not reveal a significantly larger area of contiguous optimal environment for *P. otagoense* during the LGM (Fig. 2). An apparently suitable area in North Island east coast (Hawkes Bay) might not have been reached due to physical and climatic habitat barriers. The main areas indicated as potentially suitable for *P. marginale* during the LGM (north and west North Island), were probably refugia for native forest vegetation that would have precluded these grasshoppers (Newnham *et al*., 2013). Since the Holocene, but prior to the arrival of humans about 800 years ago, most of New Zealand (87%; including nearly all lowland habitat) was under native forest vegetation; habitat not suitable for these grasshoppers. Although we determined that a suitable climate envelope (fundamental niche) existed over much of New Zealand for thousands of years, most of this area would have been unavailable to *P. marginale* (Fig. 6).

Phaulacridium marginale appears to have expanded its range into areas of open habitat as forest was replaced with native tussock grassland in eastern South Island and fernland (*Pteridium*) in North Island through human activity (Wardle, 1991; Ausseil *et al*., 2011; Perry *et al*., 2014). In contrast to climate-based models (even with vegetation and soil type included), clearing forest during the last 800 years has been the major factor determining grasshopper distribution. Before humans arrived in New Zealand there would have been little overlap between the areas with a climate envelope suitable for *P. marginale* and areas lacking forest vegetation (Fig. 6). Both *Phaulacridium* grasshoppers require open grassland/herbfield/scrub and are not found in forests. Therefore, we can infer that prior to human modification of the vegetation, *P. marginale* would have had a realised niche limited to forest edge habitat including river margins, braided rivers and coastal systems, where it does occur today. In contrast, before human activity, tussock grasslands and subalpine scrub were the only vegetation types in the current *P. otagoense* climatic range (Fig. 6), thus *P. otagoense* could have realised its full distribution based on climatic variables.

Two species, secondary contact and gene flow

Two morphologically distinct species of grasshoppers as established in their original descriptions (Westerman & Ritchie, 1984; Key, 1992) were present in our sample. Yet,

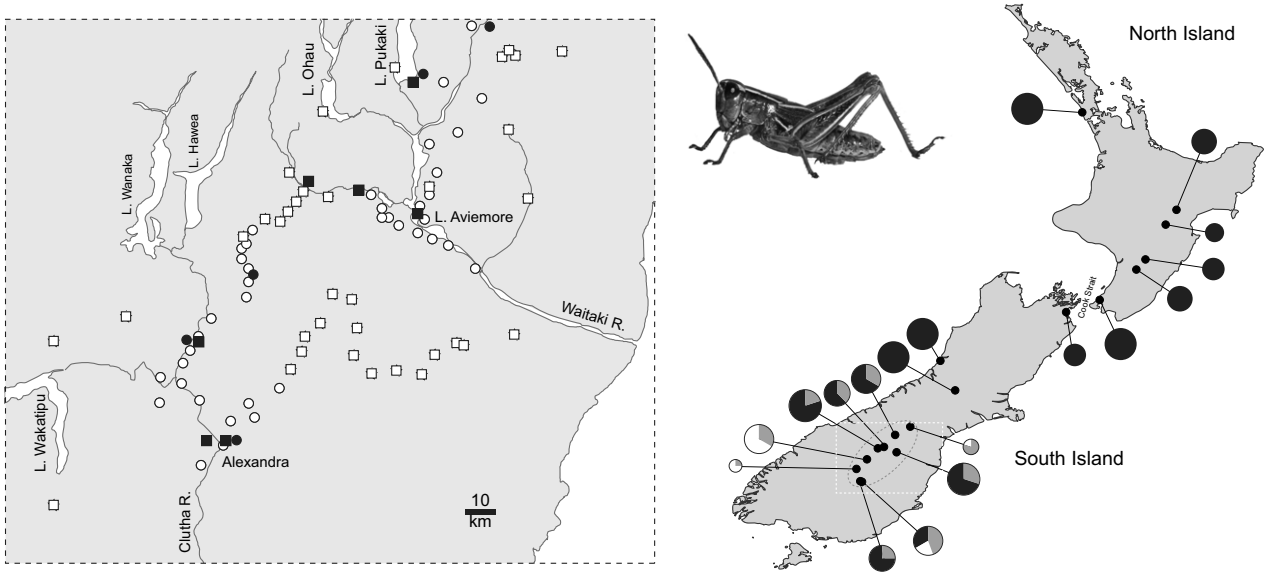

Fig. 5. Geographical distribution of *Phaulacridium* grasshoppers in New Zealand suggests recent range expansion of *P. marginale* and hybridisation in sympatry. Left: Historic distribution of *P. otagoense* (open circles) and *P. marginale* (open squares) in Central Otago and Canterbury, South Island (Westerman & Ritchie, 1984), new records reported in this study (black fill). Right: Proportional composition of population samples comprising *P. marginale* (black), *P. otagoense* (white) and hybrids (grey). Putative hybrids were inferred from mismatch between species identification, pronotum geometry and mtDNA haplotype (see Appendix S8). Intermediate forms exist only in the region of sympatry.

introgression between these two species was apparent from a mismatch between species identification (based on a diagnostic combination of three morphological traits), pronotum shape and mtDNA haplotype distribution (Fig. 3). In addition, evidence that nuclear sequences were common to the two species and diversity of variants within individuals, confirms recent inter-specific gene flow as concerted evolution normally rapidly homogenises variation among ribosomal DNA repeats (Trewick, 2001; Ganley & Kobayashi, 2007; Trewick *et al*., 2008). We found that grasshoppers of mixed ancestry were restricted to the area where the ranges of the two species overlap today. Such inter-specific introgression is most likely to occur where taxa that have diverged in isolation make secondary contact following environmental change (e.g. Durand *et al*., 2000; Stemshorn *et al*., 2011; Potts *et al*., 2014).

Phaulacridium marginale is currently widespread in New Zealand and sympatric with *P. otagoense* at three locations we surveyed. Our population samples reveal range expansion of *P. marginale* and suggest competitive exclusion within the last 30 years (Fig. 5). In contrast, *P. otagoense* is restricted to inland South Island where diurnal temperature ranges are extreme, and moisture levels low, and is now rare at sites where it was common 30 years ago (Westerman & Ritchie, 1984). Two areas in this region (Otago), Lindis Valley and Lake Dunstan, may be the last strongholds of *P. otagoense* (Fig. 6). Historical records of the distribution of *Phaulacridium* and the results of the current study, suggest that *P. marginale* is replacing *P. otagoense*. This type of advancing replacement (and introgression) has been documented in animal species pairs in various landscapes (e.g. Gill, 1980; Shapiro, 1998). In Otago New Zealand, human activity is implicated in at least three cases of genetic introgression (fish–Esa *et al*., 2000; lizards–Chapple *et al*., 2012; grasshoppers–Dowle *et al*., 2014).

The potential for the exchange of alleles among individuals, populations and higher taxonomic levels is expected in an evolving world (Vaux *et al*., 2016). However, anthropogenic habitat modification is likely to be is likely to be

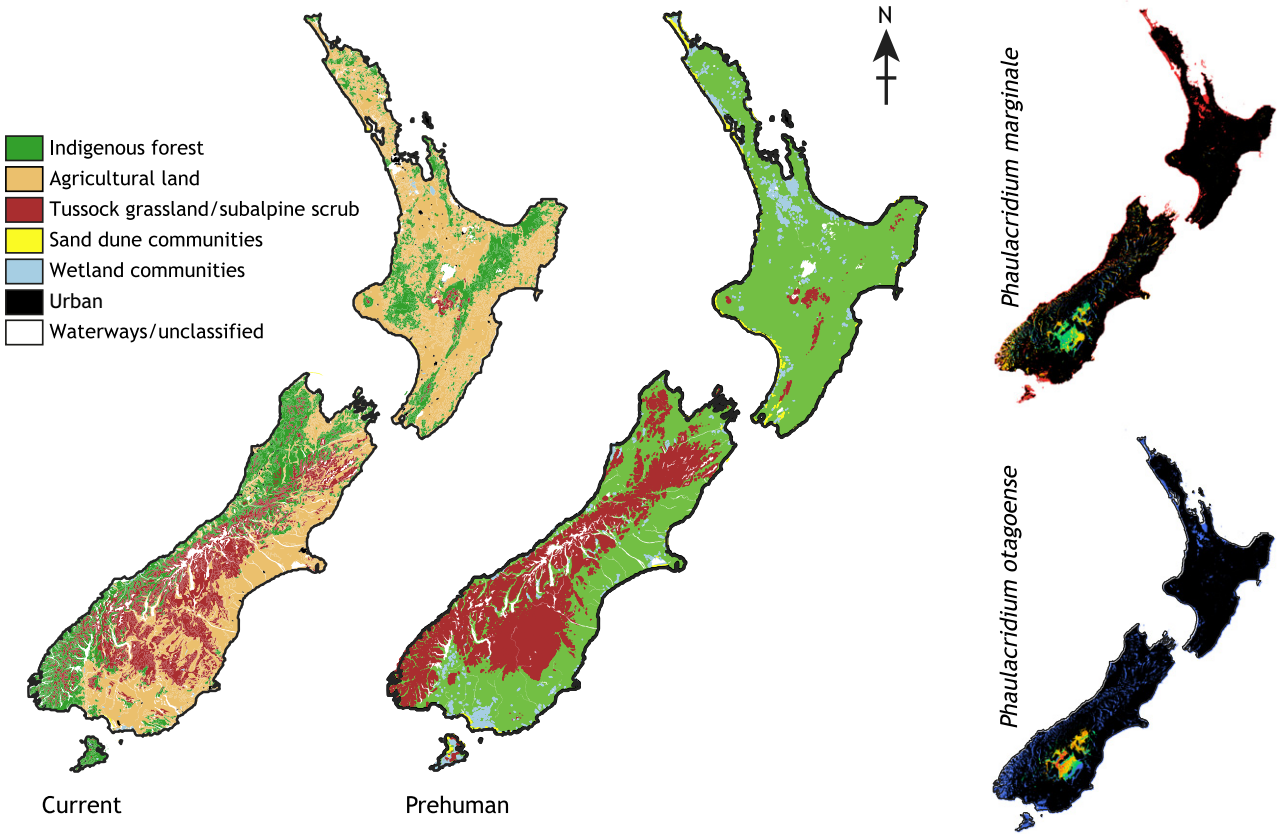

Fig. 6. New Zealand vegetation cover has changed substantially since the arrival of humans ~800 years ago. For *Phaulacridium* grasshoppers, this represents an increase in their realised niche with pre-human native forests cleared and replaced with exotic grasses and herbs. Initial expansion of native tussock grassland in south east South Island following Polynesian fires (Ausseil *et al*., 2011) has been countered by plantation forestry and agricultural intensification that is also reducing suitability of mixed exotic pastures for these and other native insects. Climate based environmental models for the two species under present conditions (from Fig. 2) were masked (black) to exclude areas naturally unavailable to *Phaulacridium* in pre-human times (Right). The mask encompasses areas with native forest cover, and elevation above 800 m a.s.l., but excludes dunes, river beds and wetlands which might have been used by *Phaulacridium*.

leading to elevated rates of secondary contact and interspecific introgression. Genomic introgression associated with alien species is expected to be aggravated by global warming (e.g. Muhlfeld *et al*., 2014), but native species also experience range shifts that increase contact (e.g. Grenouillet & Comte, 2014; Krosby *et al*., 2015). As the last substantial land environment to be colonised by people (800 years BP cf. 80 000 BP in Europe), New Zealand provides an informative system for exploring evolution of ecological responses to recent human activity. Hybridisation has been documented among New Zealand native and alien species (Morgan-Richards *et al*., 2009) with many examples linked to human activity. But, we know the interplay between gene flow and natural selection is complex and does not always result in loss of distinct traits or lineages (e.g. Dowle *et al*., 2014). Anthropogenic habitat change is strongly indicated as enabling expansion of the *P. marginale* realised niche into parts of its fundamental niche from which it was formerly excluded by native forest. Although we cannot rule out rapid ecological speciation the current distribution of phenotypic and genetic diversity suggests recent introgression of two previously partitioned species. Replacement of native forest with pasture created from introduced European herbs and grass species has been rapid and widespread in New Zealand. Ongoing agricultural 'improvement' threatens the long-term viability of endemic species throughout New Zealand and particularly in the region where *P. marginale* is increasing its range today.

Responses to future climate change are likely to involve not only range shifts and ecological competition among existing genotypes, but also gene flow that could have a major influence on evolutionary change and sustainability of biodiversity.

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: doi: 10.1111/icad.12289:

Appendix S1. Conservation resource reports used for locality records of *Phaulacridium* in New Zealand.

Appendix S2. Nineteen available climatic layers.

Appendix S3. Environmental niche model accuracy.

Appendix S4. Population sample size and location details of *Phaulacridium* grasshopper population sampled in New Zealand.

Appendix S5. (a) Morphological features used to characterise New Zealand *Phaulacridium* grasshoppers. Morphometric data were collected for: FW, hind femur width; FL, hind femur length; TL, hind tibia length; PW, pronotum width; PL, pronotum length; PA, pronotum angle; TeL, tegmina length. (b) Geometric morphometric analysis used landmarks around the margin of the pronotum (white circles).

Appendix S6. Variation at eight morphometric characters in female (A) and male (B) New Zealand *Phaulacridium* grasshoppers of two species clustered using Bayesian assignment.

Appendix S7. Classification plots for male and female New Zealand *Phaulacridium* grasshoppers using model-based clustering analysis (Mclust) with three morphometric characters, and no *a priori* identification.

Appendix S8. Individual grasshopper morphological and genetic information.

Appendix S9. Median joining network of nuclear ITS sequence variants (ITS1-5.8S-ITS2) identified from high-throughput genome sequencing of three *Phaulacridium* grasshoppers.

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