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Geographic variation in hybridization and ecological differentiation between three syntopic, morphologically similar species of montane lizards

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Abstract

To understand factors shaping species boundaries in closely related taxa, a powerful approach is to compare levels of genetic admixture at multiple points of contact and determine how this relates to intrinsic and extrinsic factors, such as genetic, morphological and ecological differentiation. In the Australian Alps, the threatened alpine bog skink *Pseudemoia cryodroma* co-occurs with two morphologically and ecologically similar congeners, *P. entrecasteauxii* and *P. pagenstecheri*, and all three species are suspected to hybridize. We predicted that the frequency of hybridization should be negatively correlated with genetic divergence, morphological differentiation and microhabitat separation. We tested this hypothesis using a mitochondrial locus, 13 microsatellite loci, morphological and microhabitat data and compared results across three geographically isolated sites. Despite strong genetic structure between species, we detected hybridization between all species pairs, including evidence of backcrossed individuals at the two sites where all three species are syntopic. Hybridization frequencies were not consistently associated with genetic, morphological or ecological differentiation. Furthermore, *P. entrecasteauxii* and *P. pagenstecheri* only hybridized at the two sites where they are syntopic with *P. cryodroma*, but not at the largest site where *P. cryodroma* was not recorded, suggesting that *P. cryodroma* may serve as a bridging species. This study reveals the complex dynamics within a three species hybrid zone and provides a baseline for assessing the impact of climate change and anthropogenic habitat modification on future hybridization frequencies.

Keywords: Australia, conservation genetics, hybridization, microsatellites, Scincidae

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Introduction

Contemporary contact between closely related species provides an opportunity to investigate the mechanisms maintaining or eroding species boundaries. The degree of genetic admixture between lineages at points of contact can range from no admixture to complete admixture, depending on numerous intrinsic and extrinsic factors, and their interaction (Singhal & Moritz 2013). Intrinsic barriers include genetic incompatibilities, which can prevent the development of viable or fertile offspring. Such incompatibilities are expected to accumulate over time due to genetic drift and/or divergent selection (Coyne & Orr 1989; Mallet 2007; Hewitt 2011), a concept central to the Bateson–Dobzhansky–Muller model of hybrid incompatibility (Turelli *et al.* 2001). To minimize energy wasted on the production of unfit

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offspring, postzygotic barriers should select for the formation of prezygotic barriers, particularly in hybrid zones, and a large literature documents such a process of reinforcement (Liou & Price 1994; Noor 1999; Turelli *et al.* 2001; Servedio & Noor 2003). Common examples of such barriers include timing of reproduction, divergence in mating preferences and mechanical incompatibility (Dobzhansky 1937). In contrast, extrinsic factors necessarily entail prezygotic barriers, comprising geographic barriers (e.g. rivers, mountains) and ecological separation (e.g. aboreal vs. fossorial; Mayr 1942).

A powerful approach to understanding factors shaping species boundaries is to compare levels of genetic admixture between species at multiple points of contact, and how this relates to intrinsic and extrinsic factors, such as genetic, morphological and ecological differentiation; yet studies that do so are relatively rare (but see Chatfield *et al.* 2010; Nice *et al.* 2013). Morphological divergence is often correlated with ecological divergence (Losos *et al.* 1998), and both may be inversely related to the likelihood of admixture. Specifically, morphological differentiation can serve as a prezygotic barrier through female preference for specific male secondary sexual characteristics and/or physical incompatibilities between taxa (Coyne & Orr 2004). Hybrid offspring that are morphologically intermediate may be unsuited to either of the parental species' ecological niches, reinforcing existing prezygotic barriers. Furthermore, differences in ecological requirements may reduce direct contact between species, decreasing opportunities for hybridization (Mayr 1942) and/or result in hybrid offspring with lower fitness (Harrison & Rand 1989; Arnold & Hodges 1995; de Leon *et al.* 2010). Consequently, species that have multiple, spatially isolated areas of geographic overlap provide an ideal system in which to examine potential factors influencing the degree of genetic admixture between species.

Although closely related lineages generally evolve and exist in allopatry (Turelli *et al.* 2001), changes in climate and habitat distribution may result in secondary contact and admixture (Rhymer & Simberloff 1996). Taxa that have recently speciated are expected to have accumulated the fewest reproductive barriers, and are consequently more likely to produce viable hybrid offspring (Orr 1995) when environmental changes bring previously isolated species into contact. When hybrids are equally fit compared with the parental species, ongoing admixture can produce a variety of outcomes. For example, hybridization can result in the formation of hybrid swarms, which have been observed primarily in fishes (e.g. Avise *et al.* 1984; Seehausen *et al.* 1997; Hasselman *et al.* 2014), but also mammals (McDevitt *et al.* 2009; Latch *et al.* 2011) and herpetofauna (Schulte *et al.* 2012; Pritchard & Edmands 2013). In rare cases, widespread hybridization can lead to the extinction of pure parentals, resulting in species collapse, as documented in three-spined sticklebacks (Taylor *et al.* 2006) and Darwin's finches (Kleindorfer *et al.* 2014). Hybridization can also be unidirectional and lead to genetic swamping of the introgressed species. This often occurs when invasive species introgress into native species (Rhymer & Simberloff 1996), and represents a significant conservation threat for numerous taxa (e.g. Allendorf & Leary 1988; Dowling & Childs 1992; Leary *et al.* 1993; Abernathy 1994; Johnson *et al.* 2010).

Here, we investigate contemporary genetic admixture and assess the relationships between the frequency of hybridization and ecological and morphological differentiation in syntopic alpine skinks (genus *Pseudemoia*). While studies on hybrid systems generally focus on two species and/or a single point of contact (but see Bogdanowicz *et al.* 2012; Fisch *et al.* 2013; Marino *et al.* 2013), we examine three species that exhibit genetic signatures of historical mitochondrial introgression (Haines *et al.* 2014) at three geographically isolated locations. All three species, *P. cryodroma*, *P. entrecasteauxii* and *P. pagenstecheri*, are morphologically similar and occupy overlapping ecological niches in the montane and subalpine regions (>1100 m above sea level) of southeastern Australia (Wilson & Swan 2013). *Pseudemoia cryodroma* is a threatened alpine specialist (DSE 2013), restricted to habitats higher than 1200 m above sea level, while *P. entrecasteauxii* and *P. pagenstecheri sensu lato* are widespread generalists that are also sympatric in lowland areas (Wilson & Swan 2013). These three species form a clade within the genus, and divergence between *P. pagenstecheri* and *P. cryodroma* plus *P. entrecasteauxii* occurred as late as 4 mya, with more recent divergence occurring between *P. cryodroma* and *P. entrecasteauxii* (Haines *et al.* 2014). We have previously provided evidence of historic hybridization between all three species pairs, with probable mitochondrial introgression from *P. pagenstecheri* into *P. entrecasteauxii* and *P. cryodroma*, as well as *P. entrecasteauxii* into its sister species *P. cryodroma* (Haines *et al.* 2014). As the previous study was based on mtDNA and multiple nuclear gene regions, it remains unclear whether contemporary hybridization occurs among these species (Haines *et al.* 2014).

In the present study, we not only test for recent admixture between species, but also explore potential drivers of hybridization. Specifically, we assess whether levels of interbreeding are consistent across three geographically isolated sites and how genetic admixture relates to the degree of morphological and ecological differentiation between species. We predict that, if present, hybridization will be highest between the recently diverged sister species *P. cryodroma* and

P. entrecasteauxii and that the level of admixture between species will be negatively correlated with morphological and ecological differentiation. By testing these predictions, this study provides rare insight into the factors influencing contemporary hybridization dynamics of three historically interbreeding species.

Materials and methods

Study sites and sampling

Pseudemoia cryodroma, *P. entrecasteauxii* and *P. pagenstecheri* are sympatric in the Australian Alps in northeastern Victoria, Australia (Hutchinson & Donnellan 1992), and researchers in the field have observed them syntopically (N. Clemann, personal observation). The three species were sampled from neighbouring mountain plateaux (Fig. 1; see Table S1, Supporting Information for sampling details) with a mosaic of snow gum (*Eucalyptus pauciflora*) woodland, alpine heathland and alpine grassland, isolated from one another by valleys of sclerophyll forests. The distance between sites ranged from 11 to 35 km. All three species were syntopic at Mount Higginbotham (HT) and the Bogong High Plains (BHP); whereas only *P. entrecasteauxii* and *P. pagenstecheri* were observed on the Dargo High Plains (DHP). Located on the border of Mount Hotham Alpine Resort and the Alpine National Park, HT comprised an area 500 m by 500 m. The BHP site (500 m by 1750 m) was in the Alpine National Park, approximately 500 m south of Mt Nelse, and on the DHP, individuals were sampled along a 10 km section of the Dargo High Plains Road and along Long Spur Track. At DHP, individuals were sampled at multiple intersections of snow gum woodland and alpine grassland, where *P. entrecasteauxii* and *P. pagenstecheri* were syntopic. Large patches of tussock grasslands, where only *P. pagenstecheri* was detected, separated these small areas of syntopy. We focused our sampling on areas of syntopy to maximize the likelihood of detecting hybrids. Therefore, we had to cover a greater overall sampling area at DHP to obtain enough samples (>20) for genetic analysis.

During the 2010–2013 breeding seasons (December to March), we collected 111, 42 and 70 *Pseudemoia* spp. from BHP, DHP and HT, respectively, through noosing and hand capture. Lizards were measured, and then either a tail tip was collected as a genetic sample and the lizard then released at point of capture, or they were retained as museum voucher specimens and liver tissue used as the genetic sample. All tissues and voucher specimens were registered at Museum Victoria (Table S1, Supporting Information). Species were identified in the field using current taxonomic identifiers: the presence/absence of vertebral stripes and paravertebral

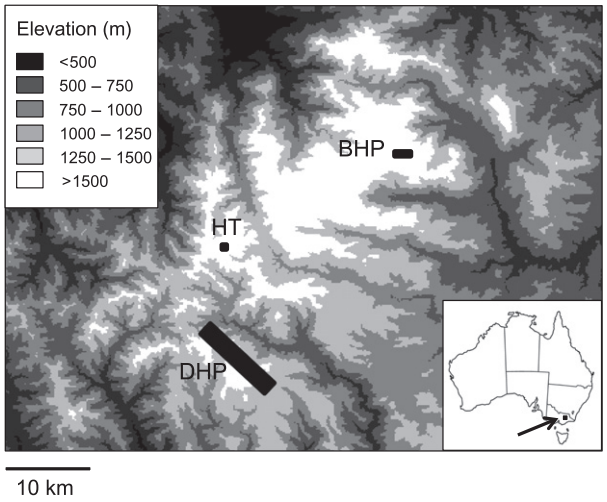


Fig. 1 Elevation map depicting sampling sites within the Victorian Alps in southeastern Australia. Site names are abbreviated: BHP, Bogong High Plains; DHP, Dargo High Plains; HT, Mt Higginbotham.

stripes, male breeding coloration and whether the midlateral stripe was well defined (Hutchinson & Donnellan 1992). The 30 individuals that could not be identified morphologically were classified as *Pseudemoia* sp. In the field, we recorded the following standard morphometric measurements: snout-vent length, distance from tip of the snout to the cloaca (SVL); head length, distance from posterior of skull to snout (HL); eye width, distance between eyes (HWE); head width between the widest part of jaws (HWJ); head depth, length at deepest part of the head (HD); interlimb length, length of the body from insertion of forelimb to insertion of hindlimb (ILL); pelvic width, width of the body at insertion of hindlimb (PW); upper forelimb, length from limb insertion to elbow (UFLL); lower forelimb, length from elbow to wrist (LFLL); forefoot, length from wrist to tip of fourth toe (FFL); upper hindlimb, length from limb insertion to knee (UHLL); lower hindlimb, length from knee to ankle (LHLL); and hind foot, length from ankle to tip of forth toe (HFL; Table S2, Supporting Information). A standard ruler was used to measure SVL to the nearest 1 mm, and digital callipers were used to measure the remaining morphological variables to the nearest 0.01 mm.

Additionally, we recorded a number of microhabitat features. We focussed specifically on microhabitat variation rather than broader environmental variables (e.g. climate, vegetation, geology) because differences between sites in broad environmental conditions, such as temperature, rainfall, elevation and vegetation, are negligible and because microhabitat variation is most likely to capture the fine-scale variation relevant to potential for admixture in these morphologically and

ecologically similar species. We recorded plant litter depth to the nearest 5 cm at the point of capture and estimated by eye the percentage of the area within a 2-m radius of the point of capture that was dominated by ground cover, shrub (vegetation <1 m high), tree (>1 m high) and rock to the nearest 5%. Tree refers to the tree trunk and low-lying branches. These ecological attributes were based on previous studies on lizard habitat use (e.g. Melville & Swain 2003; Quirt *et al.* 2006; Goodman *et al.* 2008; Teasdale *et al.* 2013). All morphometric and habitat measurements were taken by the same researcher (M. Haines).

Mitochondrial DNA sequence analysis

Genomic DNA was extracted from liver and tail tip samples using a Qiagen DNeasy Blood and Tissue Extraction Kit (Qiagen, Hilden, Germany), a GenCatch Blood and Tissue Genomic DNA Miniprep Kit (Epoch Life Sciences, Sugar Land, TX, USA) or a modified high-salt method (Aljanabi & Martinez 1997). To initially assign each individual to a given species, samples were sequenced for a 794 bp mitochondrial fragment NADH subunit 4 (ND4) and partial tRNAs using the protocol detailed in Haines *et al.* (2014). Using GENEIOUS 6.1.2 (Biomatters, Auckland, New Zealand, available at: http://www.geneious.com), sequences were aligned using the default clustering algorithm, edited and translated to amino acids. No premature stop codons were observed. Fourteen sequences had been published in Haines *et al.* (2014), and previously unpublished sequences were deposited in GenBank (Table S1, Supporting Information).

We conducted Bayesian phylogenetic analyses using MRBAYES 3.2 (Ronquist & Huelsenbeck 2003) on CIPRES Science Gateway (Miller *et al.* 2010) and maximum-likelihood (ML) analyses using PHYML 3.0 (Guindon *et al.* 2010) at http://www.atgc-montpellier.fr/phyml/. We assessed partitioning schemes and models of best fit based on Akaike information criterion (AIC) using MRMODELTEST 2.3 (Nylander 2004) and selected a GTR + Γ + I model. We included several sequences previously published in Haines *et al.* (2014), including 14 *P. cryodroma–pagenstecheri–entrecasteauxi* sequences (Table S1, Supporting Information), and sequences from the three other species within the genus *Pseudemoia* (*P. baudini*: KM263203; *P. rawlinsoni*: KM263321; *P. spenceri*: KM263326) and a closely related genus (*Niveoscincus metallicus*: KM263269) as outgroups. The Bayesian analysis consisted of two independent runs, each with four chains of Markov chain Monte Carlo (MCMC). Chains were sampled every 500 generations, and chain convergence was confirmed using an assessment of average standard deviation of split frequencies (<0.01). For the maximum-likelihood analysis, the topology was determined using NNI and approximate ratio-likelihood test (aLRT).

Primer screening and development

Fourteen microsatellite loci developed for *P. entrecasteauxii* by Stapley *et al.* (2003) were tested for cross-amplification in *P. cryodroma* and *P. pagenstecheri*. Only seven loci amplified cleanly and consistently for all three species (Table S3, Supporting Information). For these markers, fluorescently labelled dyes were attached to forward primers and a GTTTCT 'pig-tail' sequence was added to the reverse primers to reduce stuttering when scoring microsatellites (Brownstein *et al.* 1996).

As part of the current study, additional microsatellite markers were developed using 454 sequencing to supplement these published markers. One *P. entrecasteauxii* sample was sent to the Australian Genome Research Facility (AGRF, Queensland, Australia) for high-throughput DNA sequencing on 1/16 of a plate using the Roche GS FLX (454) system. A detailed sequencing protocol can be found in Gardner *et al.* (2011). Using GENEIOUS 6.1.6 (Biomatters), we searched for sequences with a minimum of 8 tetra-, penta- or hexa-repeats. Using the default parameters in the program PRIMER3 (Untergrasser *et al.* 2012), primers were designed for potential microsatellite loci. Following the approach by James *et al.* (2011), forward primers were tailed with a 454A adapter primer sequence (5′ GCCTCCCTCGC GCCATCAG 3′; Margulies *et al.* 2005), using a modified protocol of Schuelke (2000). Thirty-seven candidate loci were identified, and primers for 17 loci were tested with DNA from each of the three *Pseudemoia* spp. Loci that amplified successfully were further optimized (see below for PCR details). The seven loci that were polymorphic for the three *Pseudemoia* spp. were used, with seven of the published markers, to genotype all individuals (Table S3, Supporting Information).

Microsatellite genotyping and allelic diversity

All 223 individuals were genotyped at 14 microsatellite loci. For the loci identified by Stapley *et al.* (2003), PCRs were performed in 20 μL reactions containing 0.5 μL of each primer (10 μM), 10 μL GoTaq Hot Start Master Mix (Promega, Madison, WI, USA) and 2 μL genomic DNA. The PCRs using primers designed for this study were performed in 20 μL reactions containing 0.25 μL forward primer with 454A tail (10 μM), 0.15 μL fluorescent dye with corresponding tails (6-FAM, VIC, NED, or PET; 10 μM), 0.5 μL reverse primer (10 μM), 10 μL GoTaq Hot Start Master Mix (Promega, Madison, WI, USA) and 2 μL genomic DNA. All PCR protocols

started with an initial denaturation at 95 °C for 5 min, followed by 40 cycles consisting of denaturation at 95 °C for 30 s, annealing at temperatures ranging from 50 to 65 °C (for details see Table S4, Supporting Information) for 30 s, and extension at 72 °C for 45 s, followed by a final 5-min extension at 72 °C. The PCR products were sent to Macrogen, Inc (Seoul, South Korea), and analysed on an AB 3730 platform using a LIZ-500 size standard. Chromatograms were scored in GENEIOUS 6.1.6 (Biomatters) and visually checked for accuracy. We checked samples for identical genotypes using Microsoft Excel. In MICROCHECKER version 2.3.3 (Van Oosterhout *et al.* 2004), we checked for evidence of stutter products, large allele dropout and null alleles.

Allelic diversity was quantified for each species within sites, using only individuals with genetically pure ancestry (see Results). The number of alleles and private alleles were calculated in GENALEX 6.5 (Peakall & Smouse 2012). We determined allelic richness and private allelic richness, correcting for sample size, using HP-RARE (Kalinowski 2005). We calculated expected and observed heterozygosity and tested for Hardy–Weinberg equilibrium and linkage disequilibrium in ARLEQUIN 3.5.1.3 (Excoffier & Lischer 2010). To assess statistical significance of Hardy–Weinberg equilibrium and linkage disequilibrium results, a Bonferroni correction was used to account for multiple comparisons (Rice 1989). For each site, genetic distance between species was estimated by calculating Jost's D in GENALEX 6.5 and pairwise F_{ST} in GENODIVE 2.0b25 (Meirmans 2006).

Admixture analysis

To determine the presence of contemporary hybridization at each site, we analysed the microsatellite data from the three sites separately in the programs STRUCTURE 2.3.3 (Pritchard *et al.* 2000) and NEWHYBRIDS 1.1 (Anderson & Thompson 2002). STRUCTURE uses a Bayesian clustering algorithm to determine the most likely number of genetic clusters (K) in the data set and calculates the individual proportion of membership (Q) of each individual to each of the clusters. Individuals with a Q value close to 1 are considered pure, while those with Q values of ~0.5 for two separate clusters are likely to be F1 hybrids. In STRUCTURE, the parameters were set to allow for admixture between clusters and implemented the correlated allele frequency model. Analyses were run with a burn-in of 10^4 iterations followed by 10^6 iterations. The number of clusters (K) was set from 1 to 8 and executed five runs for each K. Results were combined in STRUCTURE HARVESTER (Earl & von Holdt 2012) and the most likely K was estimated based on where the Ln(K) plateaued (Pritchard *et al.* 2009) and the highest value of ΔK (Evanno *et al.* 2005). Outputs from STRUCTURE HARVESTER were combined in CLUMPP (Jacobsson & Rosenberg 2007).

In addition to STRUCTURE, the data were analysed with NEWHYBRIDS, which specifically calculates the probability of an individual belonging to either of the parental species and one of four hybrid classes (F1, F2 and backcrosses). As NEWHYBRIDS can only accommodate two species, for sites with three species (BHP and HT), we separately analysed individuals from each pair of parental species. Individuals were assigned to a species based on the mtDNA analysis. A model with a Jeffrey's-like prior was applied for allele frequencies and mixing proportions and implemented burn-in of 10^4 sweeps, followed by 10^6 sweeps. Runs were executed five times and repeated using Uniform priors.

As the appropriate threshold for identifying pure individuals can vary depending on factors such as allele size convergence, overall proportion of hybrids and number of loci analysed (Vähä & Primmer 2006), simulations were run to compare how accurately STRUCTURE and NEWHYBRIDS could identify both pure and admixed individuals at the commonly used thresholds of $Q \geq 0.90$ and $Q \geq 0.95$ (Burgarella *et al.* 2009; Bogdanowicz *et al.* 2012; Marino *et al.* 2013). Because all three species are sympatric throughout the Australian Alps, pure individuals could not be simulated using genotypes from nearby allopatric populations. Instead, a subset of the empirical data was used for the simulations, which has been shown to produce almost identical results (Vähä & Primmer 2006). Preliminary STRUCTURE analyses indicated that the most likely number of clusters corresponded to the number of species morphologically identified at each site (see Results); therefore, we used individuals that met the stringent threshold of $Q \geq 0.95$ for the cluster corresponding to their mitochondrial lineage to simulate pure individuals (Garroway *et al.* 2010; Tsy *et al.* 2013). Using the individuals classified as pure, we simulated genotypes for 500 individuals for each species in HYBRIDLAB (Nielsen *et al.* 2006). These simulated genotypes were used to simulate another 500 individuals for each combination of F1s and the simulated pure individuals and F1s were then used to simulate F2s, and backcrosses. To ensure that the overall proportion of simulated hybrids was comparable to that predicted for the empirical data, the number of simulated hybrids was limited to 20 randomly selected individuals from each hybrid class. The resulting data set was run through STRUCTURE using the same parameters as the original analyses, except K was set to the number of species at that site. Results were summarized in CLUMPP and visualized in MS EXCEL. The simulated data was also run in NEWHYBRIDS using the same settings as for the empirical data to compare

the accuracy of applying cut-off values of 0.90 and 0.95 for membership to either parental group or a specific hybrid class. Individuals with $Q \geq 0.90$, or $Q \geq 0.95$ for a parental category were considered to be pure for that species and all other individuals were classified as hybrids for subsequent comparisons of hybridization frequencies between sites, and in relation to ecology and morphology.

Morphological analysis

To evaluate morphological differentiation, we first tested for differences between the sexes and between sites for each species for each morphological variable. Only adults (SVL > 40 mm; Hutchinson & Donnellan 1992) were used for analyses to eliminate possible ontogenetic effects on morphology. To account for body size, measurements were regressed against SVL and the residuals were used for further analyses. Individuals were assigned to a species or classified as hybrids based on the genetic analyses. Due to small sample sizes, all hybrid classes from possible parental combinations were pooled for subsequent analyses. Univariate analyses were performed in the program R 3.0.2 (R Development Core Team 2013), applying false discovery rate corrections for multiple tests (Benjamini & Hochberg 1995). These initial univariate analyses revealed significant differences between the sexes in all species and hybrids for HL, HWJ, UFLL, LHLL and HFL ($\alpha = 0.05$); therefore, we analysed the sexes separately. As neither sex differed significantly between sites, individuals were pooled across sites for subsequent multivariate tests. A multivariate discriminant function analysis with cross-validation (PROC DISCRIM, SAS 9.3) was implemented to assess how accurately individuals could be assigned to their genetic species based on morphology.

Microhabitat analysis

Using the genetic species identifications, we assessed the degree of habitat differentiation between species at each site. Due to excess zero values in the data set (i.e. numerous individuals had 0% for one or more of litter depth, shrub, tree and rock cover), only one habitat variable (ground cover) met assumptions of normality and homogeneity of variance, even after data transformation; therefore, nonparametric analyses were performed. As initial plots of the raw data showed clear differences between sites, differences between species were tested for each microhabitat variable separately at each site using Mann–Whitney–Wilcoxon tests in R. For sites with more than two species, *P*-values were adjusted for multiple comparisons using false discovery rate. For each site, a nonparametric discriminant analysis with cross-validation (PROC DISCRIM, SAS 9.3) was also performed to assess how accurately individuals could be assigned to their genetic species based on microhabitat characteristics.

Results

Verification of species assignments

The Bayesian and maximum-likelihood analyses of the mitochondrial locus ND4 separated individuals into three well-supported clades, each representing one of the three species (Fig. S1, Supporting Information). This mitochondrial species assignment was used as an a priori hypothesis of true species identity for all subsequent analyses. The majority of mitochondrial and microsatellite identifications were congruent, and hybrids generally grouped with the mitochondrial clade corresponding to the microsatellite cluster ($Q > 0.5$). Putative F1 hybrids were randomly distributed among mitochondrial clades, suggesting bidirectional hybridization. There were four mismatches between mitochondrial and microsatellite species assignments, indicating mitochondrial introgression of *P. cryodroma* into *P. pagenstecheri*, *P. pagenstecheri* into *P. cryodroma* and two instances of *P. entrecasteauxii* into *P. cryodroma*.

Microsatellite diversity

All microsatellites were polymorphic in each species. Locus Pe24 was removed from subsequent analyses because Microchecker consistently showed an excess of homozygotes and deviation from Hardy–Weinberg equilibrium, following a Bonferroni correction, for species at the sampling location level. This result indicates the presence of null alleles, which can lead to the underestimation of true allelic diversity (Chapuis & Estoup 2007). While other loci (Pe31, Pe124, Pe197, Pe303, Pe304, Pe305 and Pe306) exhibited an excess of homozygotes and deviated from Hardy–Weinberg equilibrium, this was randomly distributed across species and localities (Table S5, Supporting Information); consequently, these loci were retained in subsequent analyses. For these 13 loci, 97.6% of alleles were successfully scored. For each species, between 1.9% and 4.5% of all possible pairwise combinations of loci were found to be in linkage disequilibrium, following a Bonferroni correction ($\alpha = 0.05$). However, linkage disequilibrium was not observed consistently between the same loci across species; therefore, no additional loci were excluded. At DHP and HT, *P. entrecasteauxii* had the greatest number of alleles and private alleles, whereas at BHP *P. cryodroma* had the highest values in these two categories (Table S5, Supporting Information). *Pseudemoia*

entrecasteauxii exhibited the highest allelic richness and private allelic richness, correcting for sample size, at each locality. Average observed heterozygosity ranged from 0.61 to 0.69 but was not consistently higher or lower for a given species or location. At each locality, both Jost's D and F_{ST} were statistically significant ($P < 0.01$) and were qualitatively the same; therefore, subsequent use of the term 'genetic distance' refers to both measures unless otherwise stated (Table 1). While genetic distance between *P. cryodroma* and *P. entrecasteauxii* was almost identical at BHP and HT, genetic distance for this pair was lower than between the other species pairs at BHP, but comparable to the genetic distance between *P. entrecasteauxii* and *P. pagenstecheri* at HT. In addition, genetic distance was always greater between *P. cryodroma* and *P. pagenstecheri* than *P. cryodroma* and *P. entrecasteauxii*.

Population structure and hybridization

STRUCTURE results indicated that the most likely number of clusters (K) was equal to the number of species recorded at that locality, with each cluster corresponding to a different species (Fig. S2, Supporting Information). Although at HT there was greater support for $K = 2$ with *P. cryodroma* and *P. entrecasteauxii* as one cluster and *P. pagenstecheri* as another cluster, when the *P. cryodroma* and *P. entrecasteauxii* cluster was analysed separately, there was highest support for splitting individuals by species into two clusters. The 95% confidence intervals (CIs) for individuals classified as pure individuals were generally between 1.00 and 0.90 and did not overlap with the CI for another cluster. For the simulated data sets, we calculated the number of pure and admixed individuals that were accurately identified at $Q \geq 0.95$ and $Q \geq 0.90$ and found negligible differences (Fig. S3 and Table S6, Supporting Information). To maximize the likelihood that individuals classified as hybrids were truly hybrids, the lower threshold was applied (Vähä & Primmer 2006). Individuals were classified as admixed between two or more species when $0.90 > Q \geq 0.10$ for multiple clusters. A lower limit of $Q \geq 0.10$ was applied to minimize the number of individuals incorrectly classified as having ancestry from all three species. Using this criterion, only 1.9% and 0.6% of simulated hybrid (F1, F2, backcrosses) individuals from BHP and HT, respectively, were misclassified.

Based on the threshold of $Q \geq 0.90$ for pure individuals, there was evidence of admixture at BHP and HT but not at DHP (Fig. S2 and Table S1, Supporting Information). The overall proportion of putative genetic hybrids was higher at HT (22.9%) than BHP (18.0%; Fig. 2), with hybridization detected between all pairs of species. The majority of hybrid individuals had Q values > 0.70 for one species and <0.30 for a second species, indicative of backcrossing (Fig. 3A). Notably, *P. cryodroma–P. entrecasteauxii* hybrids were either as common or more common as the other hybrid combinations. However, the relative proportion of other hybrid combinations was not consistent across sites. Specifically, the rarest hybrid combination at BHP was *P. entrecasteauxii–P. pagenstecheri*, which was detected in less than 2% of individuals. Nevertheless, this type of hybrid at HT was as common as *P. cryodroma–P. pagenstecheri* hybrids and comprised 4% of the data set. At both BHP and HT, one individual was morphologically identified as *P. cryodroma* yet had $Q \geq 0.10$ for all three clusters. One of the individuals was placed in the *P. cryodroma* mtDNA clade and the other in the *P. entrecasteauxii* clade. This indicates possible hybridization between two species followed by backcrossing with the third species. Additionally, four individuals identified morphologically from HT and one from BHP were assigned as genetically pure individuals ($Q \geq 0.90$) of a species that did not correspond to their morphologically assigned species. In one instance, an individual that morphologically resembled *P. pagenstecheri* but was classified as *P. cryodroma* ($Q = 0.97$) was assigned to the *P. entrecasteauxii* mtDNA clade. Notably, 17 males from BHP had a single dorsal stripe and a defined lateral

Table 1 Genetic differentiation between population pairs for each site: BHP, Bogong High Plains; HT, Mt Higginbotham; DHP, Dargo High Plains

	Pseudemoia cryodroma	*Pseudemoia entrecasteauxii*	*Pseudemoia pagenstecheri*
BHP			
Pseudemoia cryodroma	—	0.060	0.084
Pseudemoia entrecasteauxii	0.417	—	0.080
Pseudemoia pagenstecheri	0.567	0.552	—
HT			
Pseudemoia cryodroma	—	0.059	0.109
Pseudemoia entrecasteauxii	0.412	—	0.057
Pseudemoia pagenstecheri	0.653	0.374	—
DHP			
Pseudemoia entrecasteauxii	—	—	0.108
Pseudemoia pagenstecheri	—	0.706	—

F_{ST} (above diagonal) and Jost's D (below diagonal). All values are significant at $P < 0.01$.

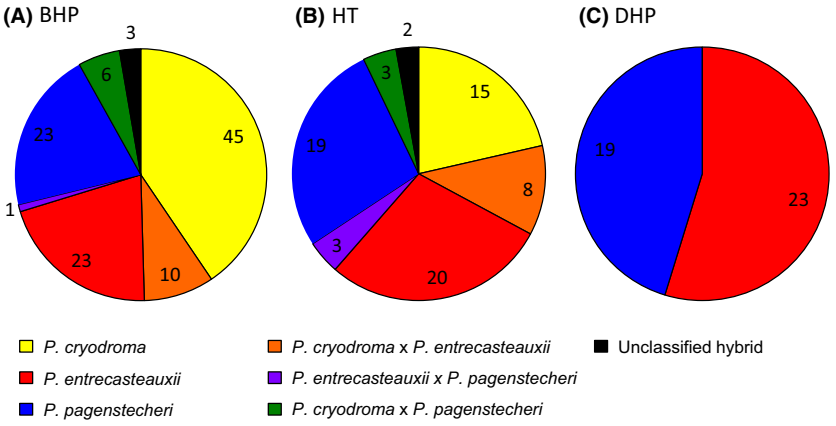


Fig. 2 Number of individuals classified as each species or hybrid cross based on individual proportion of membership (*Q*) estimated in STRUCTURE at (A) Bogong High Plains, (B) Mt Higginbotham and (C) Dargo High Plains. Classification is based on the following criteria: pure individuals had $Q \geq 0.90$ for one species, hybrid crosses had $0.90 > Q \geq 0.10$ for two species, and unclassified hybrids had either $0.90 > Q \geq 0.10$ for all three species or $0.90 > Q \geq 0.10$ for one species and $Q > 0.10$ for the other two species.

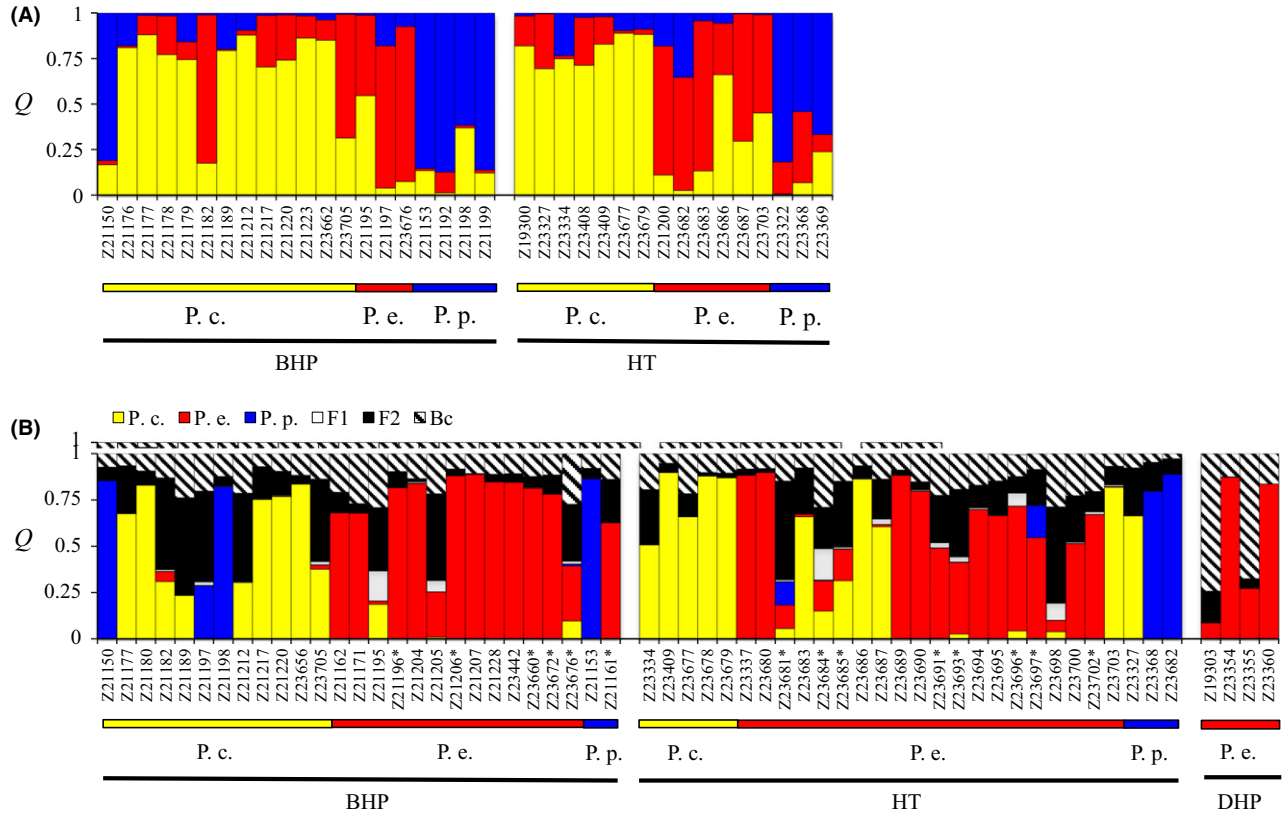


Fig. 3 Hybrid individuals identified from BHP, Bogong High Plains; HT, Mt Higginbotham; and DHP, Dargo High Plains in: (A) STRUCTURE and (B) NewHybrids. Individuals are represented by a single vertical line, with the percentage of each colour representing the individual proportion of membership (Q) for each of species: *Pseudemoia cryodroma* (yellow), *Pseudemoia entrecasteauxii* (red) and *Pseudemoia pagenstecheri* (blue). In (B), three additional categories are present: F1 hybrid (white), F2 hybrid (black) and F1 backcross (white and black stripes). Within each site, individuals are grouped by mitochondrial lineage (P.c. = *P. cryodroma*, P.e. = *P. entrecasteauxii*, P.p. = *P. pagenstecheri*). *Individual classified as a hybrid cross for two different sets of species pairs. The data presented was averaged over both analyses.

stripe, which is characteristic of *P. cryodroma*, yet they also exhibited orange/red ventral breeding coloration, which has only been reported previously in male *P. entrecasteauxii*. Fifteen of these individuals were genetically classed as pure *P. cryodroma* based on both the mtDNA and microsatellite analyses, indicating that ventral breeding coloration is not exclusive to *P. entrecasteauxii*.

The results from the NewHybrids analyses indicate an overall higher proportion of hybrids compared with

the STRUCTURE outputs. As with STRUCTURE, there was a little difference between applying the cut-offs of $Q \geq 0.90$ and $Q \geq 0.95$ to correctly distinguish between simulated pure and hybrid individuals (Table S6, Supplementary Information). Consequently, a more conservative $Q \geq 0.90$ was applied to maximize the probability of identifying true hybrids. Using uniform priors, NewHybrids identified slightly more hybrid individuals than did STRUCTURE, and considerably more using Jeffrey's-like priors. Although the NewHybrids results were broadly consistent with the results from the STRUCTURE analysis, we conservatively based on the assignment of pure and hybrid individuals on the results from the STRUCTURE analyses. Notably, the NewHybrids analysis revealed stronger evidence for F2 and backcrossed individuals than F1 hybrids; however, individuals were only assigned as F2s with more than 90% probability (Fig. 3B).

The relationship between the proportions of hybrids for each species pair was inconsistently associated with their respective genetic relatedness across sampling sites. Although genetic distance at HT was approximately 1.5 times greater between *P. cryodroma* and *P. pagenstecheri* compared with *P. entrecasteauxii* and *P. pagenstecheri*, we observed an equal number of hybrids for both pairings. However, we observed the opposite pattern at BHP, with approximately equal genetic distances between *P. cryodroma* and *P. pagenstecheri* vs. *P. entrecasteauxii* and *P. pagenstecheri*, but six *P. cryodroma–P. pagenstecheri* hybrids compared to one *P. entrecasteauxii–P. pagenstecheri* hybrid. The only pattern consistent with predictions was that as genetic distance increased between *P. entrecasteauxii* and *P. pagenstecheri*, the number of hybrids for this species pair decreased. While this pattern holds for the number of *P. cryodroma–P. pagenstecheri* hybrids, the proportion of *P. cryodroma–P. pagenstecheri* hybrids at BHP and HT is only one percentage different. Therefore, there appears to be little general concordance between genetic distance estimates between species and propensity to hybridize.

Morphological differentiation

The univariate analyses revealed significant differences in HL, HWE and all limb measurements for both sexes, and males also showed differences in HWJ and PW (Table S7, Supporting Information). In males, *P. entrecasteauxii* had larger head proportions but narrower PW compared with *P. cryodroma*. While *P. cryodroma* had longer UFLL and LFLL than *P. pagenstecheri*, both species had shorter limb measurements for five of the six limb variables compared with *P. entrecasteauxii*. Pairwise tests for females revealed similar patterns, with *P. entrecasteauxii* having relatively larger heads and longer limbs than *P. cryodroma* and *P. pagenstecheri*.

Accordingly, the discriminant function analyses revealed significant discrimination between species for both males (Wilks' $\lambda = 0.177$, $F_{39,\ 264} = 5.39$, $P < 0.001$) and females (Wilks' $\lambda = 0.284$, $F_{39,\ 189} = 2.47$, $P < 0.001$). Canonical variables (CV) 1 and 2 together explained 90.4% and 94.5% of the variation for males and females, respectively (Table 2). For males, HL, LFLL, UFLL and LHLL contributed most strongly to CV 1, while FFL contributed most strongly to CV 2, followed by the forelimb measurements. Similarly, LFLL and FFL contributed most strongly to CV 1 in females (CV 2 was not significant). In both sexes, *P. entrecasteauxii* had longer limbs compared with the other two taxa (Fig. 4). However, there was substantial morphological overlap with correct assignment of males and females to their genetic species being only 61.1% and 33.3% for *P. cryodroma*, 67.9% and 78.3% for *P. entrecasteauxii*, and 85.2% and 50.0% for *P. pagenstecheri*, respectively (Table S8, Supporting Information). Male and female hybrids were most likely to be misclassified as *P. cryodroma*.

Overall, morphological similarities between species did not correspond consistently with genetic distance or the overall proportion of different hybrid categories. The most common hybrids were between the closely

Table 2 Pooled within class canonical coefficients for canonical variables 1 and 2 for males and females from discriminant analysis of morphological variables. Morphometric variables most strongly correlated with canonical variables are in bold. Refer to text for explanations of morphological variables

	Males		Females	
Morphometric variable	Can 1	Can 2	Can 1	Can 2
SVL*	0.020	0.061	0.016	−0.374
HL	**0.457**	0.014	0.410	0.175
HWE	0.215	−0.192	0.395	0.058
HWJ	0.346	−0.086	0.192	0.134
HD	0.167	−0.155	0.152	−0.159
ILL	−0.118	0.278	−0.137	**0.487**
PW	−0.252	0.149	−0.193	0.210
UFLL	**0.401**	0.359	0.358	**0.454**
LFLL	**0.473**	**0.404**	**0.668**	**0.471**
FFL	0.184	**0.481**	**0.453**	0.307
UHLL	**0.448**	0.288	0.417	0.317
LHLL	0.287	0.042	0.436	−0.016
HFL	0.237	−0.118	0.429	0.183
F value	5.39	4.19	2.47	1.13
P value	<0.001	<0.001	<0.001	0.327
Eigenvalue	1.33	0.96	1.38	0.35
Proportion of Variance	0.526	0.378	0.755	0.190

*With the exception of SVL, morphometric variables refer to residuals calculated by regressing measurements against SVL.

related *P. cryodroma* and *P. entrecasteauxii*, but *P. cryodroma* and *P. pagenstecheri* showed least morphological differentiation. However, *P. entrecasteauxii* and *P. pagenstecheri* showed the greatest morphological differentiation in both sexes, and hybrids between this pair of species were absent at DHP, and uncommon at BHP and HT. This was most prominent in females, where no *P. entrecasteauxii* was misclassified as *P. pagenstecheri* and only 2 *P. pagenstecheri* were misclassified as *P. entrecasteauxii*.

Microhabitat differentiation

We assessed the degree of ecological separation between species based on five microhabitat characteristics: litter depth, ground cover, shrub cover, tree cover and rock cover. At all sites, univariate analyses showed significant differences between species in at least one microhabitat variable; however, differences between species were greatest at DHP and HT (Table S9, Supporting Information). Nonparametric discriminant analyses accordingly revealed the greatest habitat separation at DHP. At DHP, CV 1 explained 100% of the variation and correlated positively with ground cover and negatively with rock cover. *Pseudemoia entrecasteauxii* generally occupied areas with less ground cover and greater rock cover than *P. pagenstecheri* (Fig. 5; Table 3). However, these species did exhibit some ecological overlap, and 21.7% of *P. entrecasteauxii* and 15% of *P. pagenstecheri* were misclassified

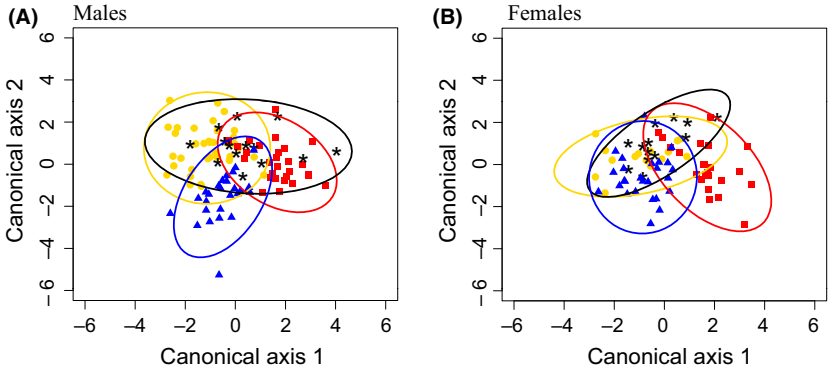
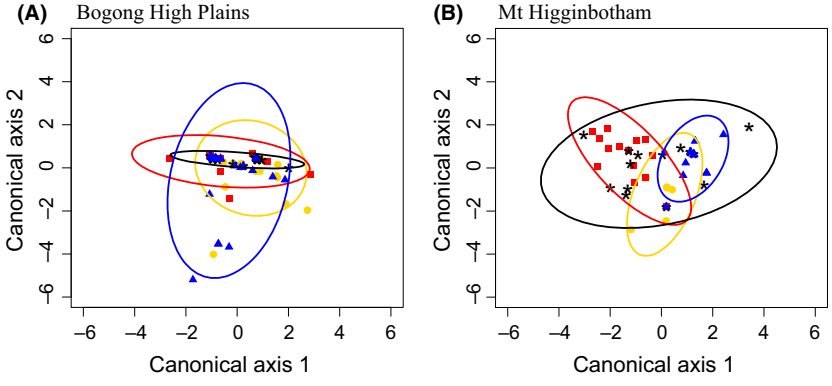


Fig. 4 Discriminant analyses based on the morphological data set for (A) males and (B) females. Individuals are represented by the following symbols: yellow circles, *Pseudemoia cryodroma*; red squares, *Pseudemoia entrecasteauxii*; black asterisks, hybrids; and blue triangles, *Pseudemoia pagenstecheri*. Ellipses represent 95% confidence levels.



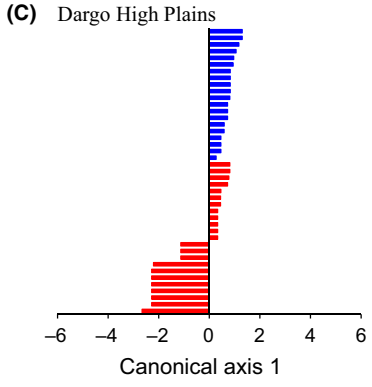


Fig. 5 Discriminant analyses based on the microhabitat data set for each site. In (A) Bogong High Plains and (B) Mt Higginbotham, individuals are represented by the following symbols: yellow circles, *Pseudemoia cryodroma*; red squares, *Pseudemoia entrecasteauxii*; black asterisks, hybrids; and blue triangles, *Pseudemoia pagenstecheri*. Ellipses represent 95% confidence levels. In (C) Dargo High Plains, individuals are represented by horizontal bars: *P. entrecasteauxii* (red) and *P. pagenstecheri* (blue).

Table 3 Pooled within class canonical structure for canonical variables 1 and 2 for BHP, HT and DHP from discriminant analysis of habitat variables. Habitat variables strongly correlated with canonical variables are in bold

Habitat Variable	BHP		HT		DHP
	Can 1	Can 2	Can 1	Can 2	Can 1
Litter depth	**−0.642**	0.226	**0.785**	0.042	0.339
Ground cover	−0.185	0.425	**0.437**	0.212	**0.965**
Shrub cover	0.452	−0.294	−0.082	0.059	−0.419
Tree cover	−0.081	0.378	**−0.664**	**0.525**	−0.128
Rock cover	−0.057	**−0.796**	0.083	**−0.727**	**−0.647**
F value	2.31	1.80	4.92	3.06	5.41
P value	0.004	0.079	<0.001	0.004	0.002
Eigenvalue	0.228	0.128	0.933	0.430	0.569
Proportion of variance	0.589	0.331	0.678	0.312	1

BHP, Bogong High Plains; HT, Mt Higginbotham; DHP, Dargo High Plains.

as each other based on habitat variables (Table S10, Supporting Information). At BHP, CV 1 and 2 collectively explained 92.1% of the variation, although only the former was significant. Higher values of CV 1 corresponded to shallower litter depth, with *P. cryodroma* occupying habitats with shallower litter depth than *P. entrecasteauxii*. Overall, discrimination was very poor at BHP and correct classification was low (*P. cryodroma* 29.0%; *P. entrecasteauxii* 47.6%; hybrids 27.3%; *P. pagenstecheri* 14.1%). By contrast, discrimination between species was much greater at HT. Both CV 1 and 2 were significant and explained a combined 99.0% of the variation. CV 1 correlated positively with litter depth and ground cover, and negatively with tree cover, whereas CV 2 correlated positively with tree cover and negatively with rock cover. The most noticeable separation was between *P. entrecasteauxii* and *P. pagenstecheri*, with the former occupying areas with lower litter depth, lower ground cover and more tree cover than the latter. Based on nonparametric discriminant analyses, hybrid individuals at BHP were most likely to be classified as *P. pagenstecheri*. Classification of hybrids was poorest at HT, where hybrids were more likely to be assigned to any of the parental species than the hybrid category.

As with morphology, misclassification rates based on ecological variables did not show a consistent correlation with genetic distance estimates or proportion of hybrids. The greatest ecological separation was between *P. entrecasteauxii* and *P. pagenstecheri* at DHP, where no hybrids were detected. By contrast, even though ecological differentiation between species was much greater at HT than at BHP, there was a higher proportion of hybrids at HT. Furthermore, the species pair with the most hybrids, *P. cryodroma* and *P. entrecasteauxii*, showed the least ecological differentiation at BHP but the highest ecological differentiation at HT. Genetic distance estimates between *P. entrecasteauxii* and *P. pagenstecheri* were highest at DHP and lowest at HT, yet misclassification rates were not substantially different between these sites.

Discussion

Our results provide evidence of strong genetic differentiation between three morphologically and ecologically similar taxa that occur syntopically within alpine and subalpine regions of southeastern Australia and have an evolutionary history of introgression (Haines *et al.* 2014). Despite strong genetic differentiation, we detected evidence of hybridization between all three species at two sites, BHP and HT, including evidence of backcrossed individuals. At the third site, DHP, only *P. entrecasteauxii* and *P. pagenstecheri* were collected, and there were no hybrids despite admixture between these two taxa at both BHP and HT. Consistent with the absence of hybrid individuals at DHP, *P. entrecasteauxii* and *P. pagenstecheri* showed the greatest morphological differentiation (all three sites combined) and the greatest genetic and ecological differentiation at DHP of any pair of species at a given site. However, contrary to predictions, genetic, morphological and ecological differentiation did not consistently correspond to the proportion of the other hybrid crosses at BHP and HT. Below, we first discuss processes that may generate the observed patterns of hybridization, and then suggest alternative explanations for geographic variation in hybridization.

Genetic differentiation despite hybridization

We detected evidence of admixture between *P. cryodroma*, *P. entrecasteauxii* and *P. pagenstecheri* at two of the three localities, yet the proportion of hybrids was generally small, particularly at BHP, and we found no evidence of hybridization between *P. entrecasteauxii* and *P. pagenstecheri* at DHP. Thus, reproductive barriers must exist; however, they are likely incomplete, as there was strong evidence of backcrossed individuals. Specifically, 19% hybrid individuals were assigned *Q* values of approximately 0.75 and 0.25 to two different populations (species) in STRUCTURE, indicative of first-generation backcrosses. Although the number of loci used was insufficient to confidently assign individuals to specific hybrid classes in NEWHYBRIDS (Vähä & Primmer 2006), there was strong evidence ($Q > 0.50$) that 23% of putative hybrids were either F2s or backcrosses. In contrast,

the NEWHYBRIDS analysis showed a maximum of $Q = 0.17$ for the F1 hybrid category. A similar pattern of fewer F1s compared with backcrossed individuals has been documented in other taxa, such as the *Ensatina eschscholtzii* salamander species complex (Alexandrino *et al.* 2005), wall lizards *Podarcis* spp. (Pinho *et al.* 2009) and spiny lizards *Sceloporus* spp. (Robbins *et al.* 2014). The low observed proportion of F1 individuals suggests potential selection against hybrids.

There is a notable absence of hybrids between *P. entrecasteauxii* and *P. pagenstecheri* at DHP, despite evidence of admixture between these species at both BHP and HT. One explanation for the disparity in hybridization between sites is that *P. entrecasteauxii* and *P. pagenstecheri* do not hybridize, or rather they do not hybridize directly. An intriguing possibility is that *P. cryodroma* serves as a bridging species. Specifically, hybrids of *P. cryodroma* and *P. entrecasteauxii* (or *P. pagenstecheri*) may hybridize with *P. pagenstecheri* (or *P. entrecasteauxii*), resulting in offspring that exhibit alleles from both *P. entrecasteauxii* and *P. pagenstecheri* despite no direct mating between pure *P. entrecasteauxii* and *P. pagenstecheri*. We did detect an individual with $Q > 0.10$ for all three species at BHP, suggesting that hybrids involving all three species do occur. Moreover, a similar case has been documented in fishes from the Colorado River Basin where direct hybridization was not detected between two of the three study species, but there was evidence of hybrids with ancestry from all three species (McDonald *et al.* 2008). Alternatively, it is possible that *P. entrecasteauxii–P. pagenstecheri* hybrid crosses exist at DHP at very low frequencies because these species are further along the speciation continuum at DHP compared with BHP and HT. This is consistent with the greater genetic and ecological differentiation between this species pair at DHP compared with the other two sites.

Geographic variation in hybridization: relationships between genetic, ecological and morphological differentiation

According to the Bateson–Dobzhansky–Muller model of genetic incompatibility, recently diverged species will have accumulated relatively fewer genetic incompatibilities compared with more distantly related species and are therefore more likely to hybridize. This pattern has been observed in numerous other vertebrates including lizards (Singhal & Moritz 2013), snakes (Tarroso *et al.* 2014) and bats (Bogdanowicz *et al.* 2012). Accordingly, we predicted that hybridization should be highest between the recently diverged sister species *P. cryodroma* and *P. entrecasteauxii* and indeed, this was the case at BHP and HT. However, the total percentage of hybrids as well as the relative proportion of each hybrid combination differed between sites. Hybridization was only observed at BHP and HT, where all three species were recorded, and was substantially higher at HT (22.9%) than at BHP (18.0%). At BHP, genetic distance between species pairs was negatively associated with the frequency of hybrids, but this was not the case at HT. Specifically, *P. cryodroma–P. entrecasteauxii* hybrids were more common than *P. entrecasteauxii* and *P. pagenstecheri* hybrids at HT, despite the two species pairs having a similar genetic distance. Thus, genetic distance between species may more closely correspond to the relative strength of prezygotic than postzygotic barriers (Coyne & Orr 1989; Turelli *et al.* 2001), particularly at HT.

We predicted that greater morphological and ecological differentiation between species would correspond to lower levels of admixture because morphological and ecological differentiation are often associated with prezygotic barriers to reproduction (Mayr 1942; Turelli *et al.* 2001; Coyne & Orr 2004). Consistent with this prediction, *P. entrecasteauxii* and *P. pagenstecheri* showed the greatest morphological differentiation in both sexes and differences in stripe patterning are most pronounced between this species pair. Furthermore, at DHP, these species exhibited the greatest ecological differentiation between any species pair at any site. Thus, ecological and morphological differentiation between *P. entrecasteauxii* and *P. pagenstecheri* may generate selection against intermediates and account for the absence of hybrids between the two species at DHP and their rarity at BHP and HT. However, in contrast to predictions, *P. cryodroma* and *P. entrecasteauxii* showed the greatest admixture, yet showed intermediate morphological differentiation across localities, and the highest ecological differentiation of any species pair at HT. Furthermore, overall microhabitat differentiation between species was substantially lower at BHP than HT; yet a higher proportion of hybrids were observed at HT. Lastly, we observed comparable ecological differentiation between *P. entrecasteauxii* and *P. pagenstecheri* at HT and DHP; however, we detected *P. entrecasteauxii–P. pagenstecheri* hybrids at HT and none at DHP. Overall, therefore, our results do not support the general prediction that hybridization should be inversely related to the degree of morphological or ecological differentiation (Mayr 1942; Turelli *et al.* 2001).

Previous work on other morphologically conserved lizards similarly found no consistent patterns between morphological differentiation and reproductive isolation (Singhal & Moritz 2013). Moreover, hybridization was roughly symmetric for all species pairs based on the mtDNA in contrast to the asymmetric introgression commonly observed between morphologically

divergent lizards (Olave *et al.* 2011; Schulte *et al.* 2012; Jezkova *et al.* 2013; Robbins *et al.* 2014; While *et al.* 2015). Interestingly, *P. cryodroma* is morphologically intermediate to *P. entrecasteauxii* and *P. pagenstecheri*, and more hybrids had *P. cryodroma* ancestry than either of the two other species. If intermediate hybrids are selected against in either parental habitat, then this should have a greater impact on *P. entrecasteauxii–P. pagenstecheri* crosses than those involving *P. cryodroma*. Nevertheless, many other pre- and postzygotic mechanisms are likely to influence the propensity to hybridize. In wall lizards (*Podarcis* spp.), where hybridization has been documented in a contact zone with no known ecological or temporal barriers (Pinho *et al.* 2009), previous studies suggest that chemosensory cues likely serve as prezygotic barriers to hybridization (Barbosa *et al.* 2006; Gabirot *et al.* 2010b). As all three *Pseudemoia* spp. have overlapping breeding seasons (Hutchinson & Donnellan 1992) and none exhibit mutually exclusive breeding coloration, the strength of chemosensory cues and other previously unmeasured sexually selected traits may determine hybridization levels. Moreover, geographic variation in such traits may further explain the observed patterns of hybridization in this study.

External factors, such as the spatial extent of suitable habitat and degree of anthropogenic habitat loss or modification, may also influence hybridization frequencies. For example, rising global temperatures have allowed both flora (Grabherr *et al.* 1994; Díaz-Varela *et al.* 2010) and fauna (Brereton *et al.* 1995; Pounds *et al.* 1999; Sinervo *et al.* 2010) to expand their ranges upward, creating overlaps with higher elevation species that are unable to realize an equivalent shift in elevation (Brereton *et al.* 1995; Parmesan 2006). Suitable subalpine habitats for *Pseudemoia* spp. are expected to shrink and/or disappear with rising average temperatures (IPCC 2013). Spatially smaller habitats provide less opportunities for microhabitat segregation, increasing the likelihood of direct contact between species and, therefore, hybridization (Rhymer & Simberloff 1996). Correspondingly, the locality with the most restricted area of suitable habitat (HT) had the highest percentage of hybrids. All samples from HT were collected within 400 m of commercial buildings, and the site itself was intersected by the region's only major roadway. Increased habitat openness has already been linked with hybridization between syntopic sister species of anoles (Jezkova *et al.* 2013). In contrast, the localities with more extensive unmodified habitat (BHP and DHP) had fewer or no hybrids. Thus, additional habitat fragmentation may increase hybridization among populations and should be considered when planning future ski resort development. Multiple, additive threats to these species (such as climate change and habitat loss and fragmentation) compound their conservation status.

Conclusion

This study provides rare insight into the current dynamics of hybridization between three montane species whose distributions are likely to shift with anthropogenic climate change (Green *et al.* 1992; Hennessy *et al.* 2003; Pickering 2007). Consistent with theoretical expectations (Turelli *et al.* 2001), the frequency of hybrids was greatest between the most recently diverged sister species pair *P. cryodroma* and *P. entrecasteauxii*. However, there was no consistent relationship between the propensity for species to hybridize and their genetic distance based on microsatellites, nor the strength of the potential prezygotic barriers examined (morphology and microhabitat). Despite clear evidence of hybridization at two of the three sites, we found strong genetic structure among species. Future research should therefore explore other potential prezygotic reproductive barriers, such as chemosensory cues. Furthermore, subsequent studies should explore the possibility that *P. cryodroma* may serve as a bridging species, facilitating hybridization between *P. entrecasteauxii* and *P. pagenstecheri* where the three species co-occur. We stress the importance of continuing to monitor admixture among these species to determine whether the frequency of hybridization shifts in response to future changes in habitat size and quality. Current suitable habitat for the montane endemic *P. cryodroma* is expected to shrink as average temperatures continue to rise and may disappear as early as 2050 (Green *et al.* 1992; Hennessy *et al.* 2003; Pickering 2007). Thus, such information is critical to understanding the evolutionary implications of hybridization among *Pseudemoia* spp. as well as the conservation management of the threatened *P. cryodroma*.

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M.L.H. contributed to the study design, conducted field sampling, performed laboratory work, analysed the data and drafted the manuscript. J.M. contributed to study design, data analysis and manuscript preparation. J.S. provided assistance with laboratory work and data analysis. N.C. contributed to project conception, interpretation, logistic support and fieldwork and provided invaluable insight into the study system. D.G.C. contributed to project conception and molecular marker development. D.S-F. contributed to the study design, data analyses and manuscript preparation. All authors provided editorial input to the final manuscript.

Data accessibility

- Morphological data, ecological data and microsatellite genotypes are available at: http://datadryad.org: doi 10.5061/dryad.kt70b.
- Mitochondrial sequences have been deposited in GenBank.
- Sampling locations are available in Supporting Information (Table S1).

Supporting information

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

Fig. S1. Bayesian phylogenetic tree based on ~800 bp of mitochondrial DNA (*ND4*).

Fig. S2. Population assignment estimated in the program STRUCTURE for the following sites: (A) Bogong High Plains, (B) Mt Higginbotham, and (C) Dargo High Plains.

Fig. S3. Population assignment for the simulated purebred and hybrid-crosses for (A) BHP, (B) HT, and (C) DHP using the program Structure.

Table S1. Locality details for all individuals, tissue numbers, voucher numbers (where specimen was taken), GenBank accession numbers, morphological identifications, and individual proportion of membership (Q) values for each cluster, with values ≥ the pure threshold (0.90) shown in bold.

Table S2. Averages (±SE) and ranges of morphological measurements for male and female *Pseudemoia cryodroma*, *Pseudemoia entrecasteauxii*, hybrids, and *Pseudemoia pagenstecheri*.

Table S3. Primer sequences, repeat motif, fragment length of PCR product, allele size range, and source for fourteen loci screened for all *P.* spp. individuals.

Table S4. Fluorescent dyes and PCR protocol details for each locus.

Table S5. Molecular diversity of *Pseudemoia* spp.

Table S6. Percent accuracy of correctly distinguishing between simulated pure and hybrid individuals, at different thresholds, using the programs Structure and NewHybrids, with both Jeffrey's and Uniform priors.

Table S7. Results of ANOVA testing for differences among species based on morphometric variables.

Table S8. Number of individuals assigned to each genetic species using a discriminant function analysis on the morphometric variables for all males and females.

Table S9. Results of Kruskal–Wallis tests for differences among species based on habitat variables.

Table S10. Number of individuals assigned to each genetic species using a discriminant function analysis on ecological variables.