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Mixed population genomics support for the central marginal hypothesis across the invasive range of the cane toad (*Rhinella marina*) in Australia

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Abstract

Understanding factors that cause species' geographic range limits is a major focus in ecology and evolution. The central marginal hypothesis (CMH) predicts that species cannot adapt to conditions beyond current geographic range edges because genetic diversity decreases from core to edge due to smaller, more isolated edge populations. We employed a population genomics framework using 24 235–33 112 SNP loci to test major predictions of the CMH in the ongoing invasion of the cane toad (*Rhinella marina*) in Australia. Cane toad tissue samples were collected along broad-scale, core-to-edge transects across their invasive range. Geographic and ecological core areas were identified using GIS and habitat suitability indices from ecological niche modelling. Bayesian clustering analyses revealed three genetic clusters, in the northwest invasion-front region, northeast precipitation-limited region and southeast cold temperature-limited region. Core-to-edge patterns of genetic diversity and differentiation were consistent with the CMH in the southeast, but were not supported in the northeast and showed mixed support in the northwest. Results suggest cold temperatures are a likely contributor to southeastern range limits, consistent with CMH predictions. In the northeast and northwest, ecological processes consisting of a steep physiological barrier and ongoing invasion dynamics, respectively, are more likely explanations for population genomic patterns than the CMH.

Keywords: amphibian, central marginal hypothesis, ecological niche model, invasive species, population genomics, species range limits

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Introduction

Understanding the factors that govern geographic range limits of species is a major focus in ecology and evolution (Darwin 1859; Haldane 1956; Mayr 1963; MacArthur 1972). The majority of species' range edges occur in areas without major physiographic barriers to dispersal (Hoffmann & Blows 1994; Parmesan *et al.* 2005; Sexton *et al.* 2009). Therefore, it is often unclear what causes the geographic range limits of many species, and why edge populations do not evolve traits that would allow them to expand their ranges (Bridle & Vines 2006; Kawecki 2008). Extensive theoretical work has been devoted to this topic, but empirical testing has lagged behind (Sexton *et al.* 2009). Testing species range limit hypotheses empirically in natural systems has become an urgent priority because global warming, exotic species invasions and habitat alteration are currently changing the distributions of many species around the world (Parmesan *et al.* 2005).

The central marginal hypothesis (CMH) is one of the major evolutionary hypotheses for species' range limits (Eckert *et al.* 2008; Sexton *et al.* 2009). The CMH is an

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extension of the abundant centre hypothesis, which predicts that habitats in the centre of a species' range will be the most favourable for supporting high population densities (Brown 1984; Sagarin & Gaines 2002). Habitat quality, population densities and connectivity among populations are consequently expected to decline towards the range periphery. The CMH describes the genetic consequences of the abundant centre hypothesis (Eckert *et al.* 2008). That is, reduced habitat quality near range edges should lead to declines in effective population sizes (N_e), which should cause higher levels of genetic drift within populations and lower geneflow rates among populations. Therefore, reduced genetic diversity and increased genetic differentiation are expected within and among edge populations relative to core populations. In turn, edge populations may lack the genetic diversity necessary to adapt to habitat conditions beyond the range edge, leading to stable range limits (Hoffmann & Blows 1994; Gomulkiewicz *et al.* 1999). Recent empirical studies testing the predictions of the CMH have been equivocal, so the generality of the CMH is unclear, particularly for invasive species (Sagarin & Gaines 2002; Garner *et al.* 2004; Eckert *et al.* 2008; Munwes *et al.* 2010; Dixon *et al.* 2013; Johansson *et al.* 2013; Micheletti & Storfer 2015; Ursenbacher *et al.* 2015). Serial founder effects and allele surfing are expected to cause reduced levels of genetic diversity at the expanding edge of an invasion wave, provided effective population sizes are small enough for genetic drift to dominate (Klopfstein *et al.* 2006; Excoffier & Ray 2008).

Species invasions provide unique, albeit unfortunate, opportunities to test evolutionary hypotheses for species' geographic range limits (Sexton *et al.* 2009; Guo 2014). The cane toad (*Rhinella* [*Bufo*] *marina*) (Linnaeus, 1758; Pramuk *et al.* 2007), native to tropical and subtropical habitats in the Americas, has become a notorious worldwide invader (Lever 2001; Kraus 2009; www.issg.org). From the mid-1800s to early-1900s, cane toads were intentionally introduced to control insect crop pests on several Caribbean and tropical Pacific islands, including Bermuda, Martinique, Barbados, Jamaica, Puerto Rico and Hawaii (Easteal 1981; Lever 2001). In 1935, 101 cane toads were collected from Hawaii and introduced to Gordonvale, Queensland, Australia, as a biocontrol agent aimed at sugar cane beetles. From 1935 to 1937, offspring from this initial Australian introduction were subsequently released and established in six sugar cane-growing regions along the east coast of Queensland (Sabath *et al.* 1981; Fig. 1). Cane toads were not an effective biocontrol agent but instead became remarkably successful invaders, spreading continuously across a range of over 1.2 million square kilometres in northern and eastern Australia, and still spreading in the northwest (Urban *et al.* 2008; Kearney *et al.* 2008; Kolbe *et al.* 2010; Fig. 1). Cane toads occupy a broader range of habitats in Australia than they do in their native range, possibly due to the lack of native Bufonid competitors in Australia (Lever 2001; Tingley *et al.* 2014). It is unclear where their highest habitat suitability or ecological core areas (Martinez-Meyer *et al.* 2012; Lira-Noriega & Manthey 2014) occur in Australia, although arid habitats lacking suitable breeding ponds and cold temperatures currently limit their distributions in inland and southern portions of their range, respectively (Sutherst *et al.* 1996; Kearney *et al.* 2008; Kolbe *et al.* 2010; Tingley *et al.* 2012; McCann *et al.* 2014). In contrast, the northwestern edge of the cane toad's range is an active invasion front, rapidly expanding at up to 55 km per year (Urban *et al.* 2008). Cane toads are of high conservation concern in Australia, as they negatively impact native biodiversity as novel toxic prey items, predators and competitors (Kraus 2009; Llewelyn *et al.* 2010; Shine 2010).

Herein, we test key predictions of the CMH, using a population genomics framework to study the ongoing invasion of Australia by cane toads. To provide context for testing the CMH, we first assess overall population structure across the range. We predict population structure to be low due to high dispersal and geneflow rates (Leblois *et al.* 2000; Estoup *et al.* 2001, 2004, 2010; Schwarzkopf & Alford 2002; Brown *et al.* 2006), or alternatively high due to serial founder effects and consequent genetic drift during their rapid range expansion (Klopfstein *et al.* 2006; Excoffier & Ray 2008). Second, the CMH predicts that reduced habitat suitability and smaller effective population sizes at the range edge will cause genetic diversity to decline from core to edge due to drift (Sagarin & Gaines 2002; Eckert *et al.* 2008). However, recent empirical findings in other systems have shown mixed support (Garner *et al.* 2004; Munwes *et al.* 2010; Dixon *et al.* 2013; Johansson *et al.* 2013; Micheletti & Storfer 2015; Ursenbacher *et al.* 2015). Third, the CMH predicts that reduced habitat suitability at the range edge will cause decreased gene flow and greater genetic differentiation among edge populations relative to those in the core (Sagarin & Gaines 2002; Eckert *et al.* 2008). Additionally, higher habitat suitability in the core will result in asymmetric gene flow from core-to-edge populations (Kirkpatrick & Barton 1997; Sexton *et al.* 2009). Alternatively, habitats may have high suitability until the edge of the range is reached, with a steep physiographic barrier at the range boundary (Hastings *et al.* 1997; Holt *et al.* 2005; Sexton *et al.* 2009). In this case, there would be no change in the levels of genetic diversity, or the rate and symmetry of gene flow, at the range edge relative to the core.

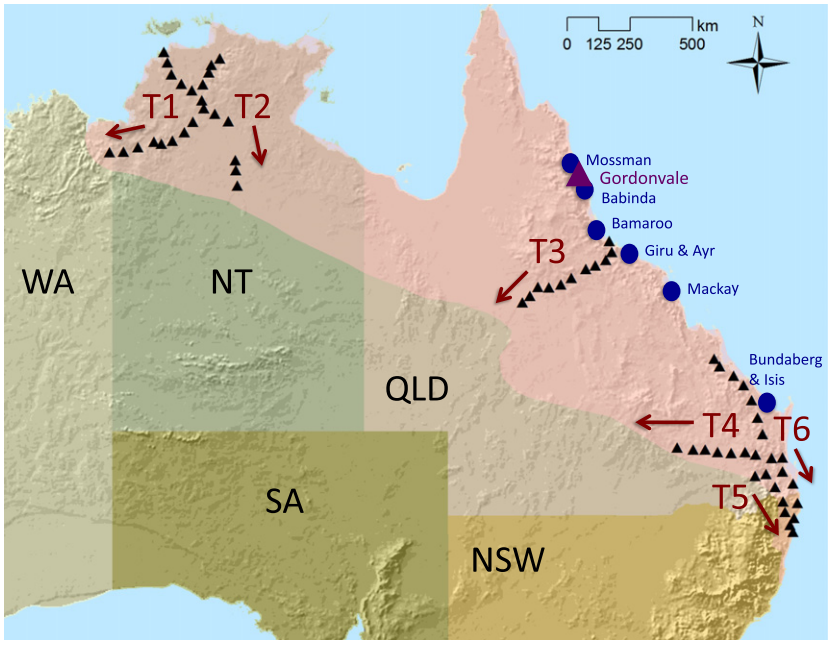


Fig. 1 Study area in Australia, including the cane toad's range, initial introduction site in 1935 (large triangle), subsequent introduction sites from 1935 to 1937 (circles), sampling sites in 2010 and 2011 (small triangles), six core-to-edge transects (T1–T6) and states (WA = Western Australia, NT = the Northern Territory, QLD = Queensland, NSW = New South Wales, SA = Southern Australia).

Materials and methods

Study area and field sampling

The cane toad's invasive range in Australia lies between −11 and −31 degrees of latitude in northern and eastern Australia (Fig. 1). It currently occupies four Australian states: Queensland (QLD), New South Wales (NSW), the Northern Territory (NT) and Western Australia (WA). Cane toads utilize a broad range of habitats in Australia, including grasslands, savannahs, dry broadleaf forests and tropical rainforests (Lever 2001). In general, precipitation decreases and aridity increases from coastal to inland sites in Australia. Vegetation tends to be denser with more tree cover along the coast, becoming sparser in inland areas. Broadly, temperature decreases along a north–south latitudinal gradient and becomes more seasonal from coastal to inland sites. Most of the cane toad's range is characterized by a summer monsoonal climate, with a warm wet season and a cooler dry season. However, the southeastern edge of the range is colder, more temperate, and receives most of its rainfall in the winter. Northern Australia has little topographic relief, whereas the Great Dividing Range runs along the east coast of Australia, providing some steep elevational gradients. The highest elevations are found in the northeastern and southeastern portions of the range, with some peaks exceeding 2000 m.

Cane toad tissue samples were collected between January and April, 2010 and 2011. Population sampling was organized along core-to-edge transects, crossing key environmental gradients hypothesized to affect the cane toad's distribution in Australia (e.g. temperature, precipitation, vegetation) at 50-km intervals. This sampling interval was chosen based on prior microsatellite work on cane toads from a small portion of their range, which showed little to no isolation by distance (IBD) up to 50 km (Leblois *et al.* 2000; Estoup *et al.* 2001, 2004, 2010). A total of 1123 individuals were sequenced from 62 populations, with a mean of 18.1 individuals per population (Table S1, Supporting information). Most individuals sampled were adults collected at breeding ponds, or from a localized area within approximately 3 km if a breeding pond could not be located. Tadpoles were collected at two locations and metamorphs at one location where few adults could be found (Table S1, Supporting information). Here, tadpoles and metamorphs were sampled from several, distant portions of the breeding pond to reduce the chances of collecting siblings.

Ecological niche modelling

We used ecological niche models (ENMs) to develop an index of habitat suitability across the Australian range of the cane toad to facilitate identification of geographic vs. ecological core and edge areas (Phillips *et al.* 2006; Micheletti & Storfer 2015). ENMs were developed using Maxent (Phillips *et al.* 2006), which uses a machine-learning algorithm that maximizes the amount of entropy in the model, or minimizes the number of constraints, to create a continuous index of habitat suitability across space. It is accurate

compared with other ecological niche modelling methods (Elith *et al.* 2006). Maxent utilizes presence-only locality data and continuous environmental data to calculate a habitat suitability index between zero and one. We gathered 3384 nonrepetitive, locality records from online biodiversity databases (Global Biodiversity Information Facility, Herpnet.org, Atlas of Living Australia), museums (Brisbane and Sydney Natural History Museums) and our own collections. Locality data spanned the cane toad's Australian range, but were highly biased to the northwestern and southeastern edges of the range. Therefore, we created a 50-km grid and intersected it with our locality points using ARCGIS 10 (ESRI, Redlands, CA, USA). We then randomly sampled one locality point per grid cell. This resulted in a less-biased data set of 264 locality points from across the cane toad's range to be used for ENM (Fig. 2).

Twenty-six continuous environmental variables were collected for ENM (Table S2, Supporting information). These variables were hypothesized to affect cane toad distributions across their range and consisted of 19 temperature and precipitation layers, vegetation (enhanced vegetation index, leaf area index, tree cover), heat load index, moisture (compound topographic index), elevation and topographic roughness (Gessler *et al.* 1995; McCune & Keon 2002; Hijmans *et al.* 2005; www.ga.gov.au, www.tern.org.au). However, some of these variables were likely to be correlated across Australia, which can reduce model accuracy (Elith *et al.* 2006). Therefore, we ran correlation tests on the variables using ENMTOOLS (Warren *et al.* 2010) and removed strongly correlated variables ($r > 0.9$) for our final ENM data set (Table S2, Supporting information).

Maxent models were run for 100 bootstrapped replicates. We used 75% of the locality data to train the models and 25% of the data to test the models, with a regularization multiplier of 1. Area under the curve (AUC) scores of the receiver-operating characteristic were calculated to assess the accuracy of the models (Swets 1988). Jackknife tests were used to determine individual variable contributions to the final models. Finally, we calculated an ecological core and a geographic core of the cane toad range in Australia (Martinez-Meyer *et al.* 2012; Lira-Noriega & Manthey 2014). The ecological core was defined as the highest median habitat suitability scores from 100 bootstrapped replicates, while the geographic core was the mean latitude and longitude of the final locality data set of 264 individuals (Fig. 2).

ddRAD sequencing

We followed the laboratory protocol of Peterson *et al.* (2012) to build ddRADseq libraries for 1123 individuals. ddRADseq was chosen to reduce the total number of SNP loci, thereby increasing the average depth of coverage per locus, given that the cane toad has a relatively large genome size of approximately 4.1 Gb (Vinogradov 1998; Peterson *et al.* 2012; www.genomesize.com). Tissue samples were stored in 80% ethanol in −80 °C freezers. DNA was extracted using Qiagen DNEasy kits. We started the ddRADseq protocol with 500 ng of DNA. We digested the DNA using *Pst*I and *Eco*RI

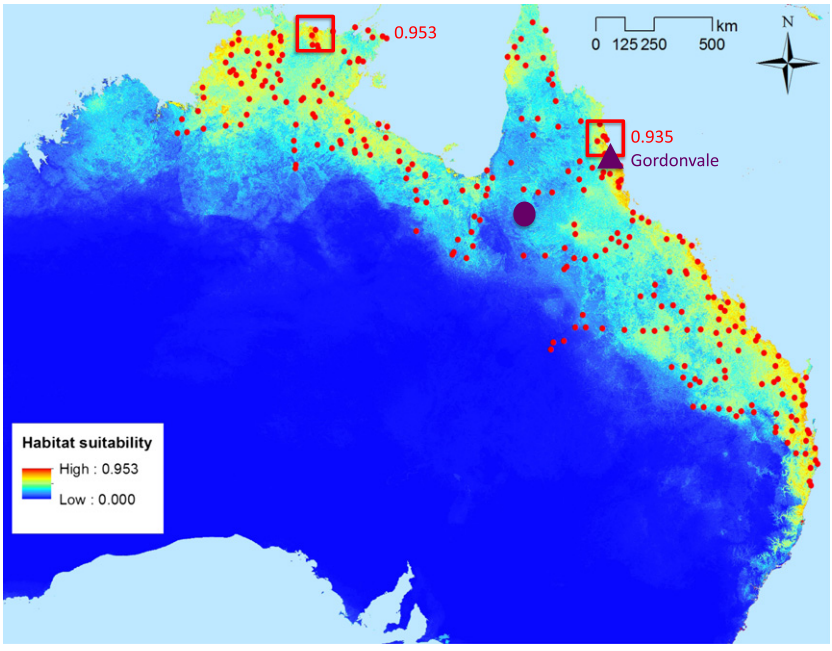


Fig. 2 Maxent model of habitat suitability including 264 localities used to build (75%) and test (25%) the model (small circles), original introduction site in Gordonvale, QLD, in 1935 (triangle), geographic centre of the range (large circle), and the highest habitat suitability values overall (0.953) and in the eastern portion of the range (0.935) (boxes). Map shows median Maxent habitat suitability from 100 bootstrap replicates.

restriction enzymes, which each recognize a different 6-bp cut site. We developed 96 P1 adapters with unique 6-bp barcodes for the *Pst*I cut sites, and nonbarcoded P2 adapters for the *Eco*RI cut sites. After ligating the adapters and pooling fragments with unique barcodes, we used a Sage Science Pippen Prep to size select 400- to 600-bp fragments. This size range was confirmed using an Agilent Technologies BioAnalyzer. We then ran 12 polymerase chain reaction (PCR) cycles using P1 primers and three uniquely indexed P2 primers, using Phusion PCR master mix, for our final ddRADseq libraries. Using a combinatorial indexing technique with unique P1 adapter barcodes and unique P2 primer indices, we multiplexed approximately 162 individuals per library, for a total of eight libraries containing 1123 individuals.

Each library was sequenced separately on a single Illumina HiSeq 2000 lane at the University of Oregon Genomics Core Facility (gc3f.uoregon.edu) using single-end 100-bp reads. We sequenced a total of 1.51 billion fragments, with 173–203 million reads per lane. We filtered out low-quality reads with the process radtags program and performed a de novo assembly and called SNPs using the denovo_map pipeline in Stacks version 1.21 (Catchen *et al.* 2013). Stacks parameter values consisted of a minimum stack depth of two to report a locus (-m argument to the ustacks program), five mismatches allowed between loci when processing an individual (-M argument to ustacks), and three mismatches allowed between loci when building the SNP catalog (-n argument to cstacks). This resulted in a total of 1.29 million SNP loci, with a mean depth of coverage of 13× (range 2–192×). We removed singletons (i.e. alleles present in only one individual), as these could be due to sequencing error. We also removed RAD loci that had >30× depth of coverage (i.e. >3 standard deviations from the mean), as well as loci with observed heterozygosity >0.5, as these loci are likely to come from paralogous regions of the genome (e.g. Hohenlohe *et al.* 2011). This filtering resulted in a data set of 337 394 putative SNP loci. Finally, we filtered our data set individually by transect and region. For this step, we removed SNPs with a minor allele frequency (MAF) <0.01 for each transect (i.e. transects 1–6, 6–14 localities per transect), MAF <0.0075 within regions (i.e. regions 1 and 3, 24 and 27 localities, respectively), and MAF <0.005 across the range (62 localities). We also removed SNPs that were present in less than 1/3 of the individuals in each transect or region. This resulted in a final data set of 24 235–33 112 SNPs per transect or region, with approximately two SNPs per 95-bp RAD locus on average (Table S3, Supporting information). For analyses requiring unlinked or loosely linked SNPs, we further filtered the data set to one random SNP per RAD locus, resulting in a final data set of 13 076–15 389 SNPs per transect or region (Table S3, Supporting information).

Population genomics

We ran two Bayesian clustering programs to estimate population structure across the range of the cane toad in Australia: ADMIXTURE (Alexander *et al.* 2009) and FASTSTRUCTURE (Raj *et al.* 2014). These analyses were individual based, as no a priori collection location information was included in either program. Both ADMIXTURE and FASTSTRUCTURE utilize a similar statistical model as STRUCTURE (Pritchard *et al.* 2000), but estimate ancestries using a faster numerical optimization algorithm. This allows them to process thousands of SNP loci in a reasonable time frame. The change in log likelihood was used to assign the number of population clusters (Evanno *et al.* 2005).

We used custom software based on LIBSEQUENCE (Thornton 2003) to estimate genetic differentiation between populations by calculating pairwise F_{ST} for each locus. Mantel tests were performed in R using the ADE4 package along each transect, comparing genetic distance (F_{ST}) to geographic distance (km) to determine whether there were significant IBD patterns. We then calculated F_{ST} per km to control for varying distances between sites when examining core-to-edge patterns along transects (Micheletti & Storfer 2015). We divided F_{ST} by the ln(distance), as F_{ST} does not increase linearly with distance indefinitely, but reaches an asymptote.

Genetic diversity within populations was estimated by Watterson's theta (θw; Watterson 1975) and theta pi (θπ; Nei 1987) for each locus. θw represents the total number of segregating sites observed (i.e. SNPs), corrected for the total number of sequences because the number of SNPs detected increases with sample size. θπ is the average number of pairwise difference across all sites. These two genetic diversity measures are proportional to the effective population size by the formula $\theta = 4N_e\mu$. We also calculated Tajima's D (Tajima 1989), which is a comparison of the total number of segregating sites to the average pairwise difference. A D of zero occurs when the total number of segregating sites equals the average pairwise difference, which is expected under neutral mutation–drift equilibrium. A negative D occurs when there is an excess of low-frequency polymorphisms, causing there to be more segregating sites relative to the average pairwise difference. This suggests the population may have experienced strong positive or purifying selection, or a recent population expansion. A positive D occurs when there are few low-frequency polymorphisms relative to intermediate frequency polymorphisms, causing the average pairwise difference to be higher than the number of

segregating sites. This suggests the population may have experienced balancing selection or recent population bottlenecks.

Finally, we used a Bayesian assignment test BAYESASS (Wilson & Rannala 2003) to estimate the symmetry of gene flow among populations, as well as NEESTIMATOR v.2 (Do *et al.* 2014) to estimate effective population sizes (N_e). Asymmetric gene flow was calculated as the proportion of migrants moving from core to edge, as well as from edge to core. BAYESASS was run for 10 000 000 Markov chain Monte Carlo (MCMC) iterations, discarding the first 1 000 000 iteration as burnin and sampling every 100 iterations. A migration rate mixing parameter (m) of 0.1 was used to optimize the acceptance rate. N_e estimates are more accurate with unlinked or loosely linked SNPs (Do *et al.* 2014), so we first filtered the SNP data sets down to a single SNP per 95-bp RAD locus. (Table S3, Supporting information). These data sets still exceeded the computer's memory requirements when computing r^2 between more than approximately 4500 SNPs. Therefore, we filtered out SNPs covered in <75% of the individuals to get a high coverage SNP data set and then randomly selected half of these high coverage SNPs resulting in a final data set of 3173–4217 SNPs per transect.

Results

Ecological niche modelling

Nine of the 26 environmental variables we tested were strongly correlated with other variables ($r > 0.9$), so we removed them from the niche models. This left 17 environmental variables in our models related to elevation and topography; vegetation density; moisture and heat load; precipitation minimum, maximum and variation; and temperature minimum, maximum and variation (Table 1). The mean AUC score across 100 bootstrapped Maxent models was 0.951, indicating high model sensitivity and specificity in predicting cane toad presence across the range.

Overall, Maxent predicted the highest habitat suitability for cane toads along the northern and northeastern coasts of Australia (Fig. 2). The highest overall habitat suitability was located at the northern coast of the NT, in a large aboriginal area called Arnhem Land (Fig. 2). This maximum habitat suitability area was approximately 1100 km northwest of the geographic mean centre of the range and 1300 km northwest of the initial point of introduction. We also calculated the highest median habitat suitability in eastern Australia, due to the high degree of geographic and genetic separation between northwestern and eastern toads. It was located only 100 km north of the initial point of introduction at Gordonvale, Queensland, and 400 km northeast of the geographic mean centre point (Fig. 2). At the individual transect scale, Maxent habitat suitability scores were generally poor predictors of genetic differentiation and diversity patterns (Figs S1 and S2, Supporting information). Therefore, we used the maximum habitat suitability indices in the northwest and east (Fig. 2) as a guide to designate more coastal and northerly sites as ecological core areas, and more inland and southerly sites as edge, for testing of CMH predictions (Fig. 1).

Table 1 Contributions of 17 environmental variables to Maxent habitat suitability models based on jackknife tests

Environmental variable	AUC with only variable	AUC without variable
Precipitation of the wettest quarter	0.871	0.922
Annual precipitation	0.865	0.921
Temperature annual range	0.819	0.921
Enhanced vegetation index	0.813	0.920
Isothermality	0.801	0.921
Minimum temperature of the coldest month	0.781	0.921
Precipitation seasonality	0.748	0.919
Precipitation of the coldest quarter	0.737	0.921
Precipitation of the driest quarter	0.735	0.921
Mean diurnal range temperature	0.732	0.921
Mean temperature of the wettest quarter	0.721	0.920
Elevation	0.667	0.919
Topographic roughness	0.642	0.920
Mean temperature of the warmest quarter	0.624	0.921
Mean temperature of the driest quarter	0.611	0.921
Heat load index	0.598	0.921
Compound topographic index of wetness	0.583	0.919

Population genomics

Bayesian clustering tests revealed little population structure across the cane toad's invasive range in Australia (Fig. 3). ADMIXTURE and FASTSTRUCTURE runs using

all 1123 individuals and 15 389 SNPs (i.e. filtered to one SNP per 95-bp RAD locus; Table S3, Supporting information) separated the northwestern invasion-front toads (transects one and two) from all of the eastern toads. We then ran ADMIXTURE and FASTSTRUCTURE on these clusters separately. The northwestern invasion-front toads showed no further population substructuring. Individuals were assigned randomly to $K = 2$ populations with no spatial pattern, suggesting the real K value was 1. The eastern toads were further divided into $K = 2$ clusters. The northeastern toads (transect three) clustered together. The southeastern toads (transects 4–6) formed the second cluster, which had more population substructure and introgression than the other two regions (Fig. 3). This suggests southeastern toad populations have more restricted gene flow, and/or more genetic drift, relative to the other two regions.

Genetic differentiation and diversity measures across the range were consistent with Bayesian clustering results, showing the highest levels of population structure in the southeast (Table 2). Genetic differentiation, as measured by pairwise F_{ST}, was low overall, suggesting high geneflow rates and/or insufficient time for genetic drift and differentiation to occur since their introduction. There was a cline of increasing genetic differentiation from the northwest to the southeast portion of the range (Table 2). This suggests gene flow is most restricted and/or genetic drift is highest in the southeast region, followed by the northeast region and the northwest invasion front. Genetic diversity measures, θw and $\theta\pi$, were low and relatively stable across the range (Table 2), consistent with a single, recent introduction of a small founder population (Sabath *et al.* 1981). Tajima's D was slightly positive across the cane toad's range (Table 2), which is indicative of either balancing selection or a recent population bottleneck. Mantel tests revealed significant IBD patterns along all transects (Table 2), confirming the spatial scale of sampling was appropriate to detect patterns of genetic differentiation and diversity.

Core-to-edge transects showed markedly different patterns of genetic differentiation and genetic diversity across the range. Genetic differentiation increased significantly along core-to-edge transects one, two and six (northwestern and southeastern regions; Fig. 4a,b,f), suggesting gene flow becomes more restricted at these range edges. There was an increasing but nonsignificant trend in genetic differentiation along transect five in the southeast (Fig. 4e), likely due to small sample size. Genetic differentiation did not increase or decrease from core to edge along transect three in the northeast (Fig. 4c), suggesting no reduction in gene flow and habitat connectivity at this range edge. Unexpectedly, genetic differentiation decreased significantly from core to edge along transect four (Fig. 4d). As inland sites tended to be warmer than coastal sites in the southeast, we further investigated the environmental factor most likely to be limiting the cane toad distributions in the south: cold temperatures. We examined the relationship between mean temperature of the breeding season and genetic differentiation. We found a significant increase in genetic differentiation from warmer to colder sites, suggesting gene flow is more restricted between cold pairs of sites than warm pairs of sites, irrespective of spatial location (Fig. 5). Genetic diversity, θw and $\theta\pi$, showed no significant patterns along any transect, so we examined core-to-edge patterns by region. The southeast region showed significant declines in both θw and $\theta\pi$ from core to edge (Fig. 6c,f). The northeast region (transect three) showed no pattern in θw and $\theta\pi$ from core to edge (Fig. 6b,e). The northwest region showed slightly increasing, nonsignificant trends in θw and $\theta\pi$ (Fig. 6a,d).

Finally, we found limited evidence of asymmetric, core-to-edge gene flow using BAYESASS, as well as strong regional differences in effective population sizes using NEESTIMATOR. Transects two, four and five had a higher proportion of migrants assigned moving from core to edge than from edge to core, whereas transects one, three and six had approximately equal proportions of core-to-edge migrants as edge to core migrants (Table 3). Estimates of N_e showed no significant patterns along core-to-edge transects, but overall N_es were largest in the northwest invasion front (123.5–172.1), intermediate in the northeast (103.0) and smallest in the cold southeast region (39.4–61.2) (Table S4, Supporting information). This marked decline in N_e from northwest to southeast provides further support for small, isolated populations in the cold southeast region, as well as surprisingly large populations at the actively expanding edge of the northwest invasion front.

Discussion

Geographic range limits of species can be caused by a combination of interacting ecological and evolutionary factors, such as demography and genetic diversity or habitat heterogeneity and gene flow (Hoffmann & Blows 1994; Parmesan *et al.* 2005; Sexton *et al.* 2009). The CMH is an evolutionary hypothesis that predicts decreased habitat suitability at the range edge will result in smaller, more isolated populations with decreased genetic diversity and gene flow relative to the core (Sagarin & Gaines 2002; Eckert *et al.* 2008). Edge populations may lack sufficient genetic diversity to adapt to environmental conditions at the range edge, which can cause either stable range limits or punctuated range expansions over time (Hoffmann & Blows

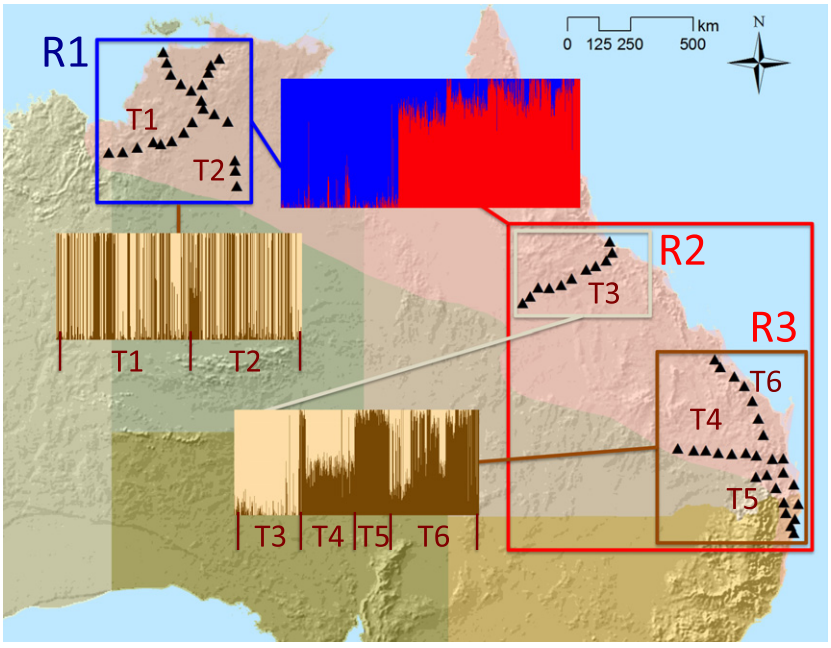


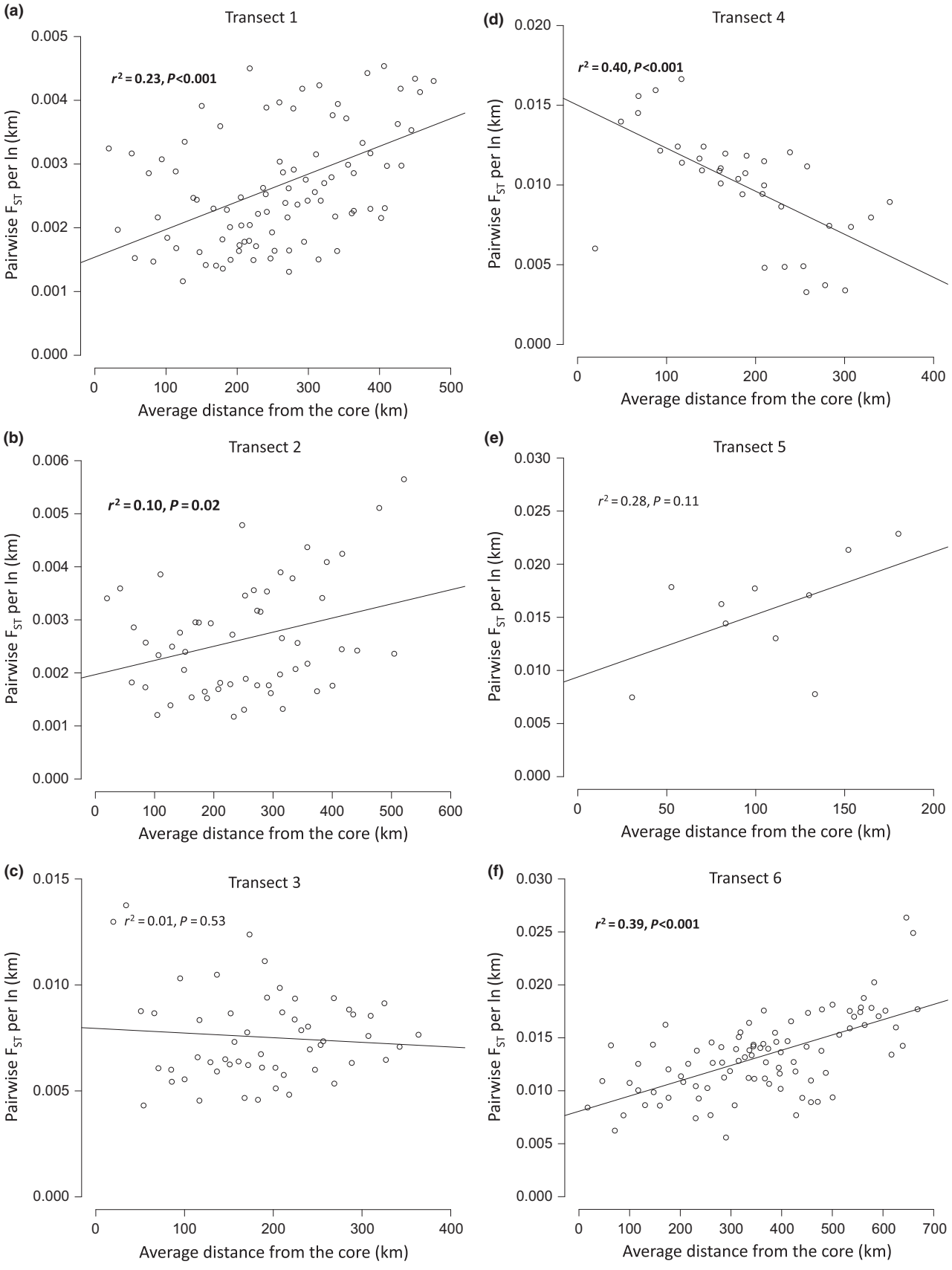
Fig. 3 ADMIXTURE population clustering plots, showing the three regions that cluster together (R1–R3), six transects within regions (T1–T6) and collection localities in 2010 and 2011 (triangles). Each line in a barplot represents an individual toad, and the colours represent the proportion of ancestry of the individual's genotype (*Q*) assigned to each population cluster (*K*).

Table 2 Genetic diversity measures, which consist of Watterson's theta (θw) and theta pi (θπ); Tajima's D; average pairwise F_{ST}; and Mantel tests for isolation by distance (IBD), for each transect and region

Transect or region	Watterson's theta (standard error)	Theta pi (standard error)	Tajima's D (standard error)	Pairwise F_{ST} (standard error)	Mantel test for IBD R^2 (*P*-value)
T1	0.00487 (0.00010)	0.00549 (0.00011)	0.438 (0.036)	0.0136 (0.0006)	0.363 (0.005)
T2	0.00434 (0.00007)	0.00515 (0.00009)	0.432 (0.029)	0.0184 (0.0008)	0.382 (0.008)
T3	0.00456 (0.00006)	0.00541 (0.00007)	0.434 (0.021)	0.0372 (0.0017)	0.704 (<0.001)
T4	0.00377 (0.00005)	0.00471 (0.00007)	0.454 (0.143)	0.0485 (0.0031)	0.572 (0.001)
T5	0.00354 (0.00008)	0.00431 (0.00010)	0.387 (0.143)	0.0708 (0.0077)	0.566 (0.040)
T6	0.00441 (0.00018)	0.00518 (0.00018)	0.371 (0.022)	0.0712 (0.0020)	0.471 (<0.001)
R1	0.00462 (0.00008)	0.00532 (0.00008)	0.424 (0.021)	0.0159 (0.0004)	0.484 (<0.001)
R2	0.00456 (0.00006)	0.00541 (0.00007)	0.434 (0.021)	0.0372 (0.0017)	0.704 (<0.001)
R3	0.00407 (0.00012)	0.00489 (0.00012)	0.400 (0.015)	0.0706 (0.0010)	0.479 (<0.001)

1994; Gomulkiewicz *et al.* 1999; Holt *et al.* 2005). For the invasive cane toad in Australia, patterns of genomic diversity and differentiation followed the predictions of the CMH in the southeastern portion of the range, but there was mixed support in the northwest and no support in the northeast (Fig. 3). In the southeast, effective population sizes were the smallest, genetic diversity decreased, and genetic differentiation increased from core to edge, consistent with the CMH (Figs 4 and 6, Table S4, Supporting information). Additionally, asymmetric, core-to-edge gene flow was found along some transects in the southeast (Table 3), potentially caused by higher quality habitats in the core relative to the edge (Mayr 1963; Kirkpatrick & Barton 1997). Cold temperatures appear to be a major limiting factor to further range expansions in the southeast (Fig. 5; Sutherst *et al.* 1996; Kolbe *et al.* 2010; McCann *et al.* 2014). While allele surfing can also cause patterns of reduced genetic diversity at expanding range edges (Klopfstein *et al.* 2006; Excoffier & Ray 2008), this explanation is less likely than the CMH in eastern Australia given that cane toads have been there the longest (Fig. 1), range limits are stable, and effective population sizes are smaller than those at the northwest invasion front

Fig. 4 Regression plots of genetic differentiation, normalized by distance between populations, represented by pairwise F_{ST} per ln (km), vs. average distance of each pair of sites to the core (km) along transects 1–6 (a–f). Transects one, two and six (a, b and f) showed significant increases in F_{ST} per ln(km) (bolded). Transect four (d) showed a significant decrease in F_{ST} per ln(km) (bolded). Transects three and five (c and e) showed no significant pattern in F_{ST} per ln(km).



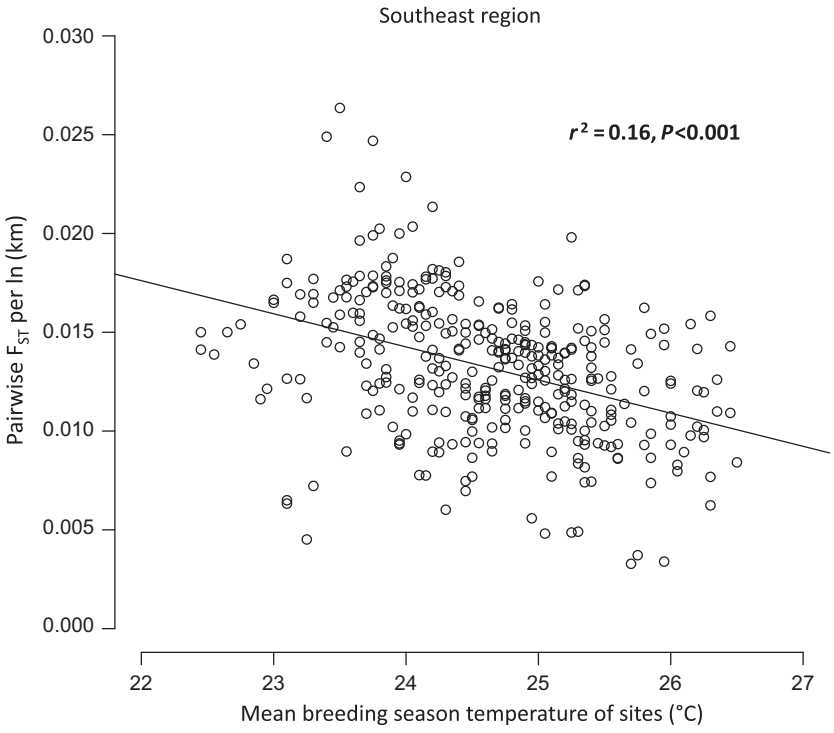


Fig. 5 Regression plot of genetic differentiation, normalized by distance between populations, represented by pairwise F_{ST} per ln(km), vs. mean temperature of the warmest quarter (i.e. cane toad breeding season in the southeast) of each pair of sites. F_{ST} per ln(km) decreases significantly from colder to warmer pairs of sites in the southeastern region.

where serial founder effects should result in the highest magnitude of genetic drift (Table S4, Supporting information).

The CMH was not supported in the northeast and showed mixed support in the northwest. Here, simpler ecological explanations of genomic patterns are more likely than the CMH. In the northeast, the rate and symmetry of gene flow and levels of genetic diversity did not change from core to edge, in spite of a strong precipitation gradient from the tropical coast to semidesert inland habitats (Table 3, Figs 4 and 6; Tingley *et al.* 2012, 2014). The inland northeast is characterized by an extreme physiological barrier, drought, combined with patchily distributed habitats that contain enough water resources for breeding and desiccation avoidance, including large rivers, cattle ponds and irrigated human developments. With sufficient habitat patch sizes and connectivity, these ecological conditions can cause abrupt species range limits with large population sizes and high connectivity all the way out to the range edge (Hastings *et al.* 1997; Holt *et al.* 2005; Sexton *et al.* 2009). At the northwest invasion front, increased genetic differentiation and asymmetric gene flow were detected from core to edge (Table 3, Fig. 4). However, effective population sizes were the largest in this region, overall levels of genetic differentiation were lowest, and genetic diversity did not decline from core to edge, but actually showed a slightly increasing, nonsignificant trend (Table 2, Fig. 6, Table S4, Supporting information). Here, the ecological dynamics of an active, ongoing species invasion are more likely to explain these genomic patterns than the CMH (Sakai *et al.* 2001; Klopfstein *et al.* 2006; Excoffier & Ray 2008; Sexton *et al.* 2009; Shine *et al.* 2012; Rollins *et al.* 2015). These mixed results highlight the value of assessing multiple transects across a species' geographic range to detect varying ecological and evolutionary processes operating at different range edges (Sexton *et al.* 2009).

Tests of the CMH

Although unfortunate, species invasions provide unique opportunities to test hypotheses for the evolution of species' range limits (Sexton *et al.* 2009; Guo 2014). However, one challenge in testing species' range limit theory is the identification of core and edge regions, particularly in invasive species that have large, noncircular geographic ranges that still expanding (Hoffmann & Blows 1994; Sexton *et al.* 2009; Micheletti & Storfer 2015). Using an ENM-based habitat suitability index, we found that the highest habitat suitability in the cane toad's Australian range was located at the northern

Fig. 6 Regression plots of genetic diversity, represented by θw (a, b, c) and θπ (d, e, f), vs. distance from the core (km) in three regions (northwest = a, d; northeast = b, e; southeast = c, f). θw and θπ decline significantly in the southeastern region (c, f) (bolded). The northwestern region (a, d) and northeastern region (b, e) showed no significant patterns in θw or θπ.

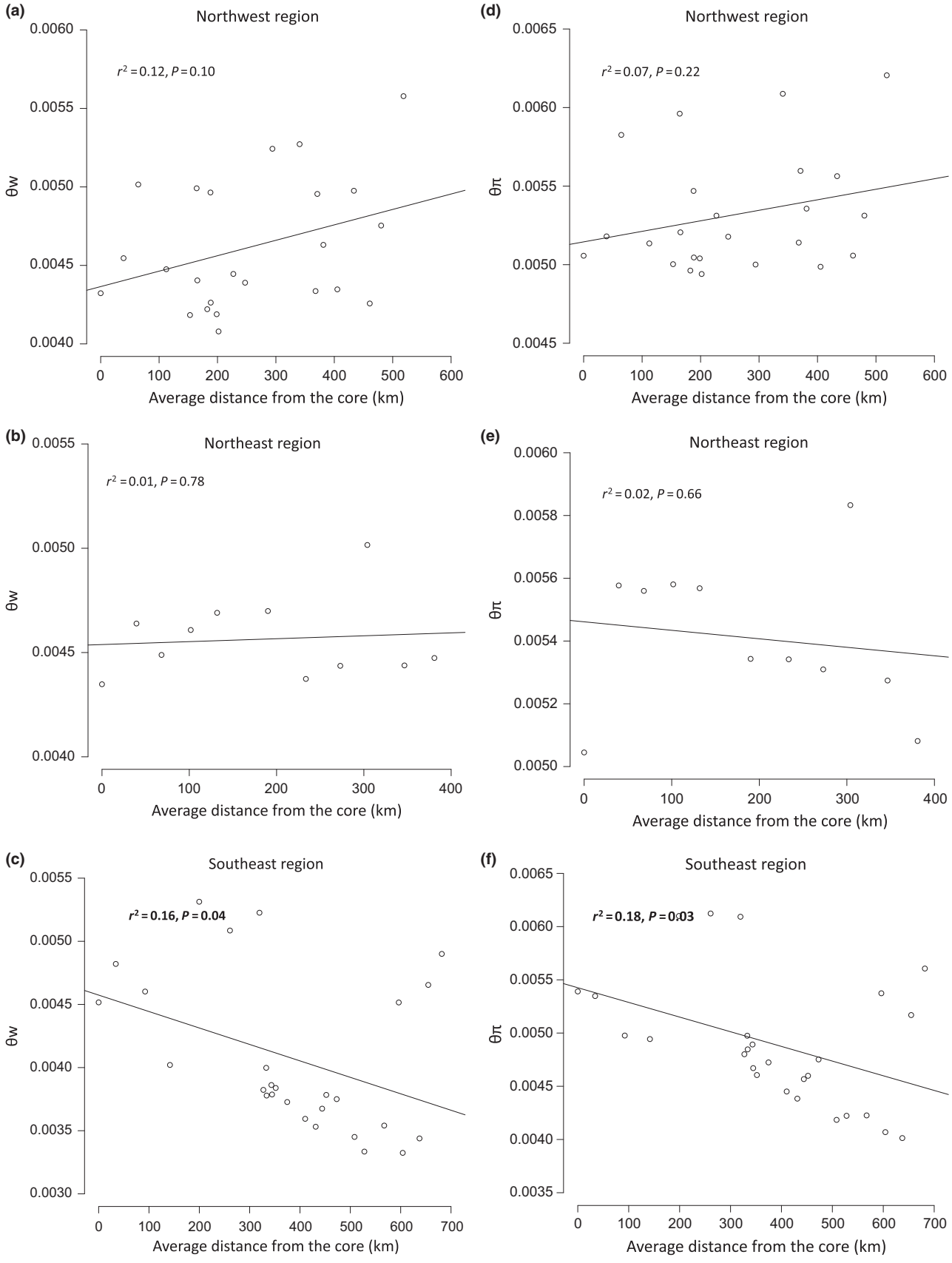


Table 3 Asymmetric geneflow estimation using the Bayesian assignment test BAYESASS. Mean migrant proportions and standard deviation across model runs were calculated from five independent BAYESASS runs for each transect

Transect	Mean proportion migrants moving in (edge to core)	Mean proportion migrants moving out (core to edge)	Standard deviation across model runs
1	0.499	0.501	0.009
2	0.438	0.562	0.006
3	0.507	0.493	0.001
4	0.400	0.600	0.003
5	0.469	0.531	<0.001
6	0.502	0.493	0.006

coast of the NT, over 1000 km west of both the geographic centre of the range and the initial point of introduction (Fig. 2). Restricting ENMs to populations in eastern Australia (Fig. 2), the highest habitat suitability was only 100 km north of the site of the initial introduction in tropical, northeast Queensland (Fig. 2). The geographic mean centre of the range had a relatively low habitat suitability index score (0.283; Fig. 2). This low score highlights the importance of using a habitat-based suitability measure to define the ecological core of the range, rather than a simple geographic approximation of the range centre as most empirical range limit studies have done in the past (Garner *et al.* 2004; Munwes *et al.* 2010; Martinez-Meyer *et al.* 2012; Dixon *et al.* 2013; Johansson *et al.* 2013; Lira-Noriega & Manthey 2014; Micheletti & Storfer 2015; Ursenbacher *et al.* 2015). ENM-based estimates of the core largely matched our intuitive predictions that more coastal and northerly regions would have higher habitat suitability (Fig. 2). However, it was not apparent which of the northwestern coastal populations should be assigned as core in analyses that combined transects one and two until ENM was performed (Figs 3 and 6).

The southeastern region had the smallest effective population sizes, and it was the only portion of the cane toad's range where we found significant declines in genetic diversity from core to edge (Fig. 6, Table S4, Supporting information). Cold temperatures are a likely contributing factor, as colder sites were significantly more genetically isolated than warmer sites (Fig. 5). Therefore, small population sizes and lack of genetic diversity at cold edge sites likely limit cane toad range expansion to the south. However, ongoing global climate change may allow for future cane toad range expansions in the south by increasing local temperatures, resulting in higher habitat suitabilities and thus potentially a shallower environmental gradient to adapt (Kawecki 2008; Sexton *et al.* 2009).

In the northeast, there was no evidence of increased genetic differentiation, decreased genetic diversity, or asymmetric gene flow, as predicted by the CMH (Figs 4 and 6, Table 3; Eckert *et al.* 2008). Here, simpler ecological explanations appear more likely than the CMH. Cane toads are limited by a steep physiological barrier at this range edge, consisting of extreme aridity and drought resulting in a lack of breeding ponds and high desiccation risk (Tingley *et al.* 2012, 2014). However, there are also large inland rivers (e.g. Flinders River), permanent cattle ponds and irrigated human developments located near this range edge. Arid habitats that contain permanent moisture to avoid desiccation, and at least occasional standing water for breeding, likely allow dispersal and gene flow to remain high within otherwise inhospitable edge environments. When suitable habitat patches are of sufficient size and connectivity, steep ecological barriers to dispersal can result in stable levels of gene flow and genetic diversity all the way out to the range edge (Hastings *et al.* 1997; Holt *et al.* 2005; Sexton *et al.* 2009).

The northwest region is still an expanding invasion front (Kearney *et al.* 2008; Urban *et al.* 2008), so the CMH is an unlikely explanation for genomic patterns of diversity and gene flow. Instead, continual establishment of founder populations at the invasion front can lead to scattered populations with low gene flow among them (Sakai *et al.* 2001; Klopfstein *et al.* 2006; Excoffier & Ray 2008; Shine *et al.* 2012). Therefore, edge populations are expected to be more genetically isolated, with asymmetric gene flow from larger, more contiguous core populations (Fig. 4, Table 3; Kirkpatrick & Barton 1997; Sakai *et al.* 2001; Eckert *et al.* 2008). Surprisingly, effective population sizes were not smaller than the core in this recently invaded region, and genetic diversity did not decline but rather increased slightly from core to edge (Fig. 6a,d, Table S4, Supporting information). Abundance of cane toads appears to be highest in newly colonized areas and decreases in long-colonized areas, perhaps due to reduced food availability or increased parasite loads (Freeland 1986; Shine 2010). High habitat suitability in the northwest (Fig. 2), and potentially the evolution of increased dispersal phenotypes in an expanding invasion front (Alford *et al.* 2009; Phillips *et al.* 2010; Shine *et al.* 2012; Rollins *et al.* 2015), may also help explain the high overall levels of gene flow, genetic diversity and large effective population sizes found in this region (Table 1, Fig. 3, Table S4, Supporting information).

Historical demography

Overall, cane toads in Australia showed low levels of population genomic structure and genetic diversity across their vast invaded range (Table 2, Fig. 3). These

genomic patterns are consistent with numerous ecological studies documenting the toad's extreme dispersal abilities relative to other amphibians (Schwarzkopf & Alford 2002; Brown *et al.* 2006; Shine 2010) and a well-documented history of founder effects during their invasion (Easteal 1981; Sabath *et al.* 1981; Lever 2001). Mean Tajima's D values across loci were slightly positive (Table 2). Whereas range expansions can cause Tajima's D values to be negative by increasing the number of low-frequency mutations through allele surfing and genetic drift (Klopfstein *et al.* 2006; Excoffier & Ray 2008), strong population bottlenecks can cause Tajima's D values to be positive by eliminating low-frequency mutations. Low θ values across the cane toad's geographic range can be caused by strong population bottlenecks as well (Table 1), as θ is proportional to past effective populations sizes and mutation rates ($\theta = 4N_e\mu$). Prior studies using 12 microsatellites from a smaller portion of the cane toad's Australian range have also shown evidence of population bottlenecks (Estoup *et al.* 2001, 2004, 2010).

Cane toads in Australia have a well-documented invasion history of multiple population bottlenecks and founder effects (Easteal 1981; Sabath *et al.* 1981; Lever 2001). In the mid-1800s, cane toads were introduced from their native South American countries of Guyana and French Guiana to the Caribbean islands of Bermuda, Martinique, Barbados and Jamaica. In 1920 and 1923, they were introduced from Barbados and Jamaica to Puerto Rico. In 1932, they were introduced from Puerto Rico to the Hawaiian Islands. Finally in 1935, 101 cane toads were collected from just a few localities on the island of Oahu and introduced to Gordonvale, Queensland, Australia. Offspring from this initial Australian introduction were subsequently released and established in six sugar cane-growing regions along the east coast of Queensland from 1935 to 1937 (Fig. 1; Sabath *et al.* 1981). Thus, cane toads have only been present in Australia for approximately 80 generations, assuming a minimum 1-year generation time (Phillips & Shine 2005). Our population genomic results suggest that high dispersal and gene flow, as well as strong and relatively recent population bottlenecks, are dominating the effects of isolation and genetic drift due to serial founder effects and allele surfing (Klopfstein *et al.* 2006; Excoffier & Ray 2008) during the cane toad's rapid invasion of Australia.

Conclusion

Cane toads have been extremely successful invaders in Australia, quickly becoming one of the largest and most damaging amphibian invasions in the world (Lever 2001; Kraus 2009; Shine 2010; www.issg.org). This vast invasion is ongoing, as the northwestern edge of their range is still an actively expanding invasion front. ENMs predict cane toad spread along the northern and western coasts into the southern part of Western Australia (Kearney *et al.* 2008; Kolbe *et al.* 2010). Moreover, the southeastern range edge is likely to expand further with warming temperatures due to global climate change. We found that the CMH and cold temperatures are likely contributors to the toad's current range limits in the southeast, but not in the other two regions. Therefore, future management efforts in the southeast should consider targeting populations with high levels of adaptive genetic diversity. In arid, interior portions of the range, management measures should limit toad access to moisture and standing water wherever possible, such as cattle ponds and irrigated developed areas. Overall we found mixed support for the role of the CMH in evolving new range limits in this infamous and ongoing species invasion.

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D.R.T. collected field data, performed laboratory work, performed population genomic and ecological niche modeling analyses and wrote the manuscript; B.E. performed population genomic analyses; L.S. designed research and directed field work; P.A.H. designed laboratory and population genomics research and directed laboratory work; R.A.A. directed field work; A.S. directed the project, designed research, contributed to data analyses and contributed to writing the manuscript; and all authors contributed input to draft and final versions of the manuscript.

Data accessibility

ddRADseq and locality data used in genomic and niche modelling analyses are available at Dryad (doi: 10.5061/dryad.5ps15).

Supporting information

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

Table S1 Field samples collected from 62 cane toad populations; including sample size, transect membership, and life stage.

Table S2 Correlation matrix of 26 environmental variables considered for ecological niche modeling with Maxent.

Table S3 Number of SNP loci per transect or region retained after filtering, as well as the number of sampling sites and number of individuals sequenced per transect or region.

Table S4 Effective population sizes (N_e), as well as means and standard errors across each transect, estimated using NEESTIMATOR v.2 (linkage disequilibrium method).

Fig. S1 Regression plots of genetic differentiation, normalized by distance between populations, represented by pairwise F_{ST} per ln(km), vs. average Maxent habitat suitability index of each pair of sites along transects 1–6 (a–f).

Fig. S2 Regression plots of genetic diversity, represented by θw (a, b, c) and θπ (d, e, f), vs. Maxent habitat suitability index in three regions (northwest = a, d; northeast = b, e; southeast = c, f).