

Genomic costs without functional consequences in short-range endemics trapped in evolutionary refugia

Running title: Comparative genomics of evolutionary refugia

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Abstract

Species with small geographic ranges are often concentrated in evolutionary refugia, landscapes that support viable populations through significant climate oscillations. Under long-term stable environments and with small population size, species are predicted to evolve narrow climatic tolerances, lose genetic diversity and accumulate harmful mutations. Together, these predictions suggest that short-range endemic species may be highly vulnerable to extinction. Here, we investigate the genomic and evolutionary consequences of persistence in refugia using a radiation of Australian geckos (*Gehyra* spp.) as a model system. With 82 genomes from eight species, spanning short-range and widespread pairs, we demonstrate the long-term persistence of short-range endemics at sustained small population size. Short-range endemics have lower genomic diversity and higher inbreeding, but do not show excess fixation of harmful mutations compared to their widespread relatives. We find a relative deficit of high-impact mutations in the most inbred species, consistent with purging of strongly deleterious variation. Despite restricted distributions, the physiological tolerances of short-range endemics are highly plastic, and similar to widespread species. Together, these results suggest that species in evolutionary refugia may be no more intrinsically susceptible to extinction than their widespread counterparts. Despite this, short-range species remain highly vulnerable to extinction via extrinsic factors, including catastrophic environmental events.

Introduction

Why some species have very small ranges in comparison to their close relatives is a long-standing question in both ecology and evolutionary biology¹. Species with small geographic ranges are often concentrated in evolutionary refugia², landscapes that have retained suitable conditions over recent millennia despite large-scale climatic oscillations^{3–6}. The Climatic Variability Hypothesis predicts that species living in more stable environments, such as evolutionary refugia, may become adapted to a more restricted environmental range than species experiencing variable environments^{7–11}. It may therefore be that short-range endemic (SRE) species have evolved more specialised niches than their widespread relatives^{8,12,13}. Paradoxically, this means that SRE species may become ‘trapped’ in evolutionary refugia, with reduced capacity to persist in the long-term.

In addition to potential reduced environmental tolerance, population genetic theory predicts that populations with sustained small effective size (N_e) will rapidly lose genetic diversity and that mildly deleterious mutations will increase in frequency through genetic drift^{14–16}. This is expected to reduce their ability to respond to selection for climatic stress, thereby reducing their adaptive capacity under changing environments^{7,17}. SRE¹⁸ species also have an extrinsically elevated extinction risk due to low population numbers and restricted geographic ranges¹⁹, and are often lacking information for adequate conservation assessment²⁰. The combined effect of having a specialised niche, low genetic diversity, and an elevated mutational load should further place SRE species at elevated risk of extinction via intrinsic factors^{21,22}. Despite this, we observe many such species at present-day. There has been much recent discussion in the literature about the ability of small populations to purge deleterious mutations via selection (e.g.^{23,24}), but whether evolutionary refugia foster the required demographic conditions²⁵ for genetic purging remains untested.

80

81 To understand the evolutionary and genomic impact of long-term restriction to evolutionary
82 refugia, we need to determine whether species we observe as ‘short-range endemics’ today
83 have a quantifiable history of small effective population size (N_e) across multiple glacial cycles,
84 or previously had large N_e and have recently contracted to small geographic ranges. Coalescent-
85 based historical demographic reconstructions using genomic data present a powerful
86 opportunity to test these hypotheses, with analyses based on multiple whole-genome
87 haplotypes (e.g. MSMC2²⁶) providing the temporal resolution required for working at
88 phylogeographic timescales (i.e. from as old as 1 million generations before present, to as
89 recent as 40 generations before present²⁷). Further leveraging genomic data, we can determine
90 the consequences of contrasting demographic histories on genomic diversity, inbreeding, and
91 the burden of deleterious mutations. By conducting synergistic physiology experiments, we
92 can also determine whether SRE species have reduced physiological tolerance, indicating a
93 more specialised environmental niche, compared to their widespread relatives.

94

95 As a case study to address these questions, here we focus on the Australian *Gehyra* radiation,
96 a species-rich group of geckos that exhibit extensive variation in range size, including closely
97 related widespread and SRE species with overlapping distributions. Geckos are particularly
98 prone to having small ranges, with many species known only from their type locality²⁰. This
99 reflects their generally low dispersal ability and propensity to adapt to specific habitats, often
100 isolated rocky ranges within woodlands or deserts. Recent range-wide genetic analyses of
101 widespread species of Australia's tropical *Gehyra* revealed many phylogenetically divergent
102 lineages with no mixing in sympatry or parapatry^{28,29}. This has led to the recognition of several
103 new species, including rock-specialists with very small geographic ranges^{30–32} and reduced
104 genetic diversity³³. These species are distributed in the Top End and Kimberley of northern

Australia, regions with highly seasonal rainfall and pronounced changes in climate from the cold-dry last glacial maximum (LGM), through the wetter early Holocene^{34,35}. Desiccation resistance is thought to be an important adaptive trait in reptiles, with previous work showing that evaporative water loss varies with environmental conditions³⁶. Consistent with the Climatic Variability Hypothesis, short-range *Gehyra* may be adapted to a more restricted environmental range than their widespread relatives, and therefore show contrasting patterns in key adaptive traits, like evaporative water loss. This unusually well-studied Australian radiation, with multiple SRE and widespread lineages, presents an ideal system with which to explore the consequences of long-term confinement to evolutionary refugia.

Building on this wealth of existing knowledge about the *Gehyra* radiation, we generate whole genome data from 82 individuals across eight species, anchored by long-read chromosome-level reference genomes, and combine these with results from physiology experiments. Using these multiple data sources, we investigate the evolutionary and genomic consequences of long-term confinement to evolutionary refugia to determine whether extinction risk in short-range endemics is exacerbated by niche specialisation, loss of genomic diversity and accumulated genetic load.

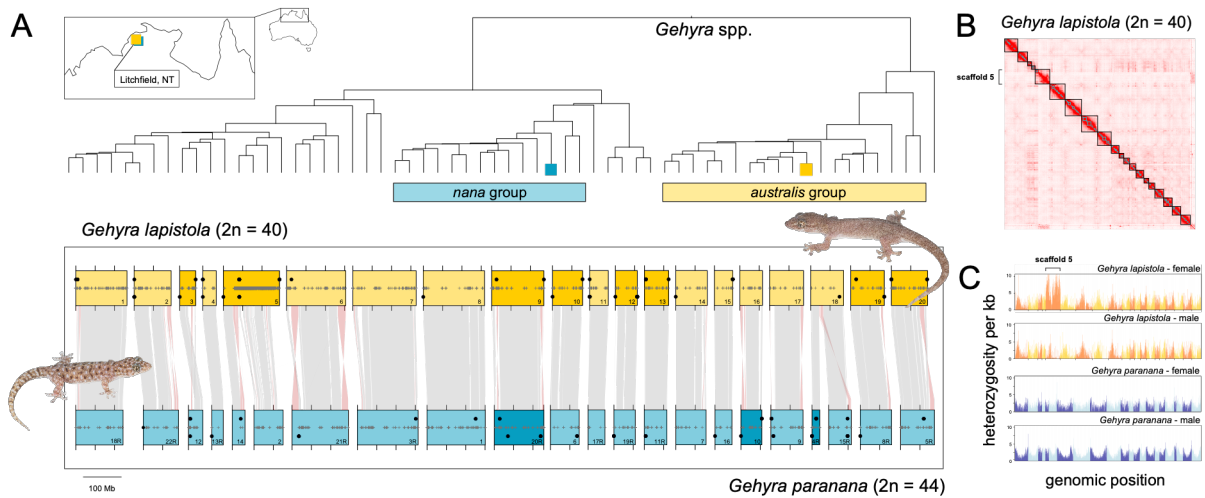
Results

Two new chromosome-scale reference genomes for Australian geckos

We assembled new, chromosome-scale reference genomes for two species of Australian geckos in the *Gehyra* radiation, *G. lapistola* (2n = 40) from the *australis* group, and *G. paranana* (2n = 44) from the *nana* group (Fig. 1), originating from Litchfield National Park in the Northern Territory (Table S1). These are among the first reference genomes from Australian representatives of Family Gekkonidae³⁷, and are highly contiguous (>97.5%

scaffolded onto chromosome-scale contigs) and with high BUSCO completeness (Table S2). In both cases, our reconstructed number of chromosome-level scaffolds matches expectations from cytology^{38,39}. The ancestral *Gehyra* karyotype is expected to be $2n = 44$ ³⁹, and we therefore hypothesise that two major chromosome-level rearrangements (including the event involving the putative sex chromosome in *G. lapistola*; see below) are the result of chromosomal fusions in the lineage leading to *G. lapistola* (Fig. 1A).

Our results indicate the presence of differentiated ZW sex chromosomes in the *G. lapistola* genome. Hi-C contact maps show long-range interaction between scaffold 5 and several small, unplaced scaffolds in the *G. lapistola* assembly 5 (Fig. 1B), but no such interaction in the *G. paranana* genome (Fig. S1). In addition, our analysis of the distribution of genome-wide heterozygosity across chromosomes in males and females shows elevated heterozygosity at scaffold 5 in female *G. lapistola* only, but no evidence for sex-based heterozygosity differences in *G. paranana* (Fig. 1C). Leveraging novel whole-genome resequencing across all species generated in this paper, we show that this sex difference in heterozygosity on scaffold 5 extends to all species within the *koira* subclade of the *australis* group, to which *G. lapistola* belongs, and is consistently absent in the *nana* group (Fig. S2). This elevated heterozygosity is likely the result of both Z and W reads mapping to scaffold 5 in *G. lapistola*. Our analyses of published reduced representation sequencing data from a broader set of species further support this, showing elevated heterozygosity on scaffold 5 in females of all species within the *koira* subclade, and no evidence for this pattern on scaffold 5 in the *australis* group subclade containing *G. arnhemica*, *G. australis*, *G. lauta* and *G. gemina* (Fig. S3). Together, these results support the presence of novel, differentiated ZW chromosomes in females within the *koira* subclade of the *australis* group.



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157

158 **Figure 1. New chromosome-scale reference genomes for two range-restricted Australian**

159 **geckos.** A) 20 scaffolds of *G. lapistola* (top, yellow) represent >97% of the assembly, and 22

160 scaffolds of *G. paranana* (bottom, blue) represent >98.5% of the assembly. Blocks of synteny

161 between the genomes (i.e. collinear, complete BUSCO regions) are indicated in grey (same

162 strand) and red (inverse strand) shading. Filled circles indicate predicted telomeres and crosses

163 indicate contig joins after Hi-C scaffolding. Darker shaded scaffolds are potential telomere to

164 telomere chromosomes. Scaffold numbers in each respective assembly are labelled, and are

165 sorted in the plot based on the scaffold name and orientation in the *G. lapistola* assembly (‘R’

166 indicates reverse orientation in synteny plot). Inset phylogeny³³ shows the evolutionary

167 position of *G. paranana* in the nana group, and *G. lapistola* in the australis group, and inset

168 map shows the sampling location of both individuals from Litchfield National Park, Northern

169 Territory. B) Hi-C contact map of *G. lapistola* showing 20 scaffolded contigs, and the presence

170 of long-range contact association between scaffold 5 and small, unplaced contigs (bottom right

171 corner of the contact map), which is typical of partially collapsed, partially differentiated neo-

172 sex chromosome (see Fig. S1). C) The distribution of female and male genome-wide

173 heterozygosity in *G. lapistola* (orange and yellow) and homologous scaffolds in *G. nana* (light

and dark blue), both ordered by their position in the *G. lapistola* reference genome. The female *G. lapistola* shows elevated heterozygosity on scaffold 5, while the male does not. Photos by Scott Macor.

Low genome-wide diversity and variable inbreeding history in short-range endemics

Levering these two new high-quality genomes as references to anchor our analyses, we successfully generated high-quality, short-read genome sequence data from 82 individuals from eight species of *Gehyra* distributed across northern Australia for population-level analysis, in the *australis* (Fig. 2A) and *nana* (Fig. 2B) groups. These included five short-range endemic (SRE) species, and three widespread species, spanning several orders of magnitude in range size (Fig. 2C), with an average genome-wide sequencing depth of 17X (Table S1). After filtering, we retained 11.15 million clade-level, cohort-called variable sites for the *australis* group, and 42.35 million variable sites for the more diverse *nana* group (Table S3), each cohort representing the diversity of four species. Based on these cohort-called SNPs, all samples clustered within their expected species groups in PCA space (Fig. 2A, Fig. 2B).

Within each group, widespread species had significantly higher individual heterozygosity (at neutral sites) compared to their SRE relatives across all contrasts (Fig. 2D). Species in the *nana* group tended to have higher heterozygosity compared to species in the *australis* group (Fig. 2D), consistent with previous work based on reduced representation sequencing comparing genetic diversity in these groups³³. Average pairwise relatedness values within all species was negative, indicating that they do not share more alleles than expected by chance (Table S4), reflecting the geographic spread of sampling.

Within the *australis* group, individuals of the short-range endemics *G. ipsa* and *G. lapistola* had a significantly higher proportion of their genomes contained within runs of homozygosity (F_{ROH}) than the widespread species *G. koiria* (Fig. 2E), indicating greater inbreeding in SRE species. Individuals of the SRE *G. calcitectus* also had higher genomic inbreeding than *G. koiria* on average, but this difference was not significant. In the *nana* group, genomic inbreeding (F_{ROH}) in the SRE *G. paranana* was also higher than its widespread relative *G. nana*, although not significantly so (Fig. 2E). In contrast to expectations, average F_{ROH} of the SRE *G. girloorloo* was lower than its widespread relative *G. kimberleyi* (Fig. 2E), indicating an absence of long-term inbreeding in *G. girloorloo* despite its current small distribution. Overall, species in the *australis* group tended to have higher F_{ROH} (Fig. 2E) and larger ROH block sizes than species in the *nana* group (Fig. S4). This indicates more recent and extensive inbreeding in the *australis* group, especially for short-range endemic species. Several *G. lapistola* individuals had ROH blocks of >15 Mb in size (Fig. S4), suggesting particularly recent inbreeding in this species. We found a significant positive correlation between range size and species-level mean heterozygosity, but not between range size and mean F_{ROH} (Fig. 2F), suggesting that present-day range size is not a consistent predictor of the extent of historical inbreeding.

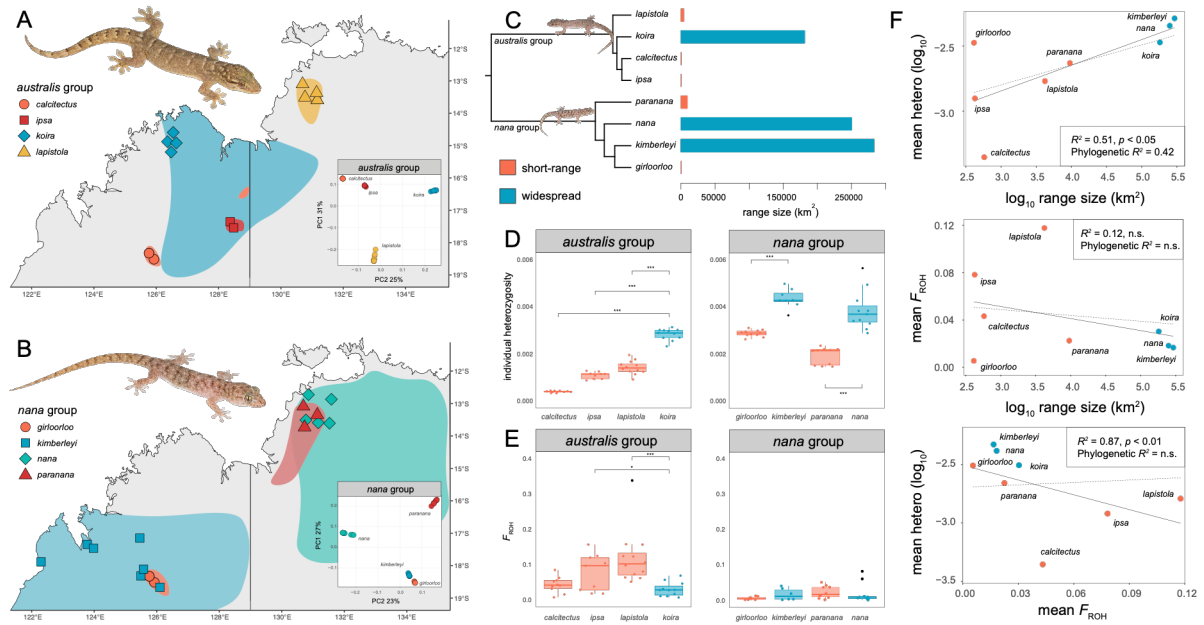


Figure 2. Low genomic diversity and high inbreeding in short-range endemics, compared to their widespread relatives. A) Species distributions (coloured shading), sampling locations (coloured shapes) and principal components analysis from whole-genome resequencing of four focal species in the *australis* group, and B) four focal species in the *nana* group. C) Relative range sizes of short-range (red) and widespread species (blue). D) Average genome-wide individual heterozygosity across autosomes, E) the proportion of the genome in runs of homozygosity (F_{ROH}), with significant differences between species within each clade indicated by square brackets (* = $p < 0.05$, *** = $p < 0.001$), with outliers shown as black points. F) Linear models showing the relationship between range size, mean species heterozygosity and mean species F_{ROH} . Solid lines represent standard linear models and dashed lines represent phylogenetic linear models. Heterozygosity and range size are log transformed in panel F. Photos by Scott Macor.

Sustained small population size in short-range endemics, with one recent contraction

Historical demographic reconstructions using MSMC2 show that in most cases (i.e. *G. lapistola*, *G. ipsa*, *G. calcitectus*, *G. paranana*), species with present-day small ranges have a long-term history of small effective populations size (Fig. 3; Fig. S5), consistent with persistence in small area evolutionary refugia through climate changes. In contrast, one of our short-range species *G. girloorloo* shows evidence for a long-term history of fluctuating effective population size with recent demographic decline. This is consistent with our above results of relatively low F_{ROH} in *G. girloorloo*, suggesting that this species has experienced a very recent population contraction. The historical demographic response seen in *G. girloorloo* is similar to that of widespread species *G. koira*, *G. nana* and *G. kimberleyi*, with N_e expanding after the wetter mid-Holocene (Fig. 3).

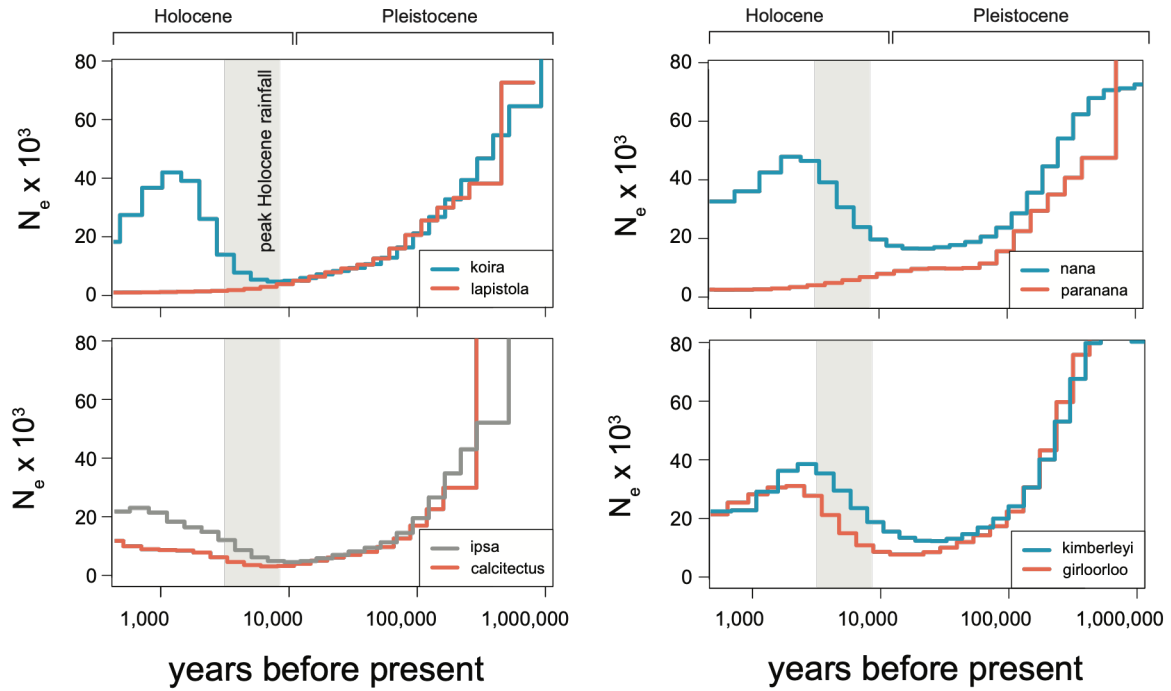


Figure 3. A long-term history of small effective population size in short-range endemics.

Historical demographic history inferred using MSMC2 across all eight *Gehyra* species, with x-axes truncated at 400 years before present. Widespread species are shown in blue, and short-range species are shown in red or grey, noting that the demographic reconstruction of *G. girloorloo* does not reflect a long-term history of small N_e . Plots are visualised with a generation length of 2.5 years⁴⁰, and mutation rate from⁴¹.

275 *Evidence for genetic purging via inbreeding in short-range endemic species*

276 Overall, species in the *nana* group show higher segregating genetic load, i.e. burden of
277 putatively harmful mutations, than those in the *australis* group (Fig. 4; Fig. S6), consistent with
278 higher neutral diversity and lower inbreeding in the *nana* group (Fig. 2). There is a significant
279 positive relationship between segregating mutations of all three predicted functional impact
280 categories (high, moderate and low impact; Fig. 4A; Fig. S8) and range size, with the number
281 of putatively deleterious (high or moderate) segregating mutations scaling with the number of
282 neutral mutations (LOW). For example, *G. calcitectus* has the lowest number of segregating
283 mutations in all impact categories (Fig. 4A), and has the lowest genome-wide diversity overall
284 (Fig. 2). There is a corresponding negative correlation between fixed, species-specific
285 alternative homozygous mutations and range size in all impact categories, with short-range
286 species having higher fixed derived alleles than widespread relatives (Fig. 4B). There is no
287 correlation between the ratio of fixed derived high-to-low impact mutations (Fig. 4B) or fixed
288 derived high+moderate-to-low impact mutations (Fig. S7) and range size, either in linear or
289 categorical comparisons, suggesting that populations with present-day small ranges in this
290 study do not have an elevated rate of fixation of putatively deleterious mutations compared to
291 those of their widespread relatives (Fig. 4). Given that present-day range size is an inconsistent
292 predictor of past demographic history (e.g. in *G. girloorloo*), and that long-term population
293 dynamics are more consequential to genetic load than present-day range size, we also tested
294 for a relationship between F_{ROH} and the ratio of fixed, derived high-to-low and moderate-to-
295 low impact mutations. We found that the genomic inbreeding coefficient, or F_{ROH} , significantly
296 predicted the ratio of high-to-low impact mutations, where species with higher inbreeding (i.e.,
297 higher F_{ROH}) had a lower relative number of fixed high impact mutations compared to fixed
298 low impact mutations. For example, *G. lapistola* had the highest average F_{ROH} and also had a
299 relative deficit of high impact mutations, while *G. girloorloo* had the lowest F_{ROH} and a relative

excess of fixed, high impact mutations (Fig. 4C). In contrast, there was no evidence for a relationship between the ratio of moderate-to-low impact mutations and F_{ROH} , suggesting that inbreeding has played a role in purging the highest-impact, putatively deleterious mutations. We found that while F_{ROH} is not significantly correlated with present-day range size (Fig. 2F), it is significantly correlated with the harmonic mean of historical N_e in linear models, although not in phylogenetic linear models (Fig. 4E). Notably, long-term N_e was not significantly correlated with the ratio of high-to-low or moderate-to-low impact mutations (Fig. S8).

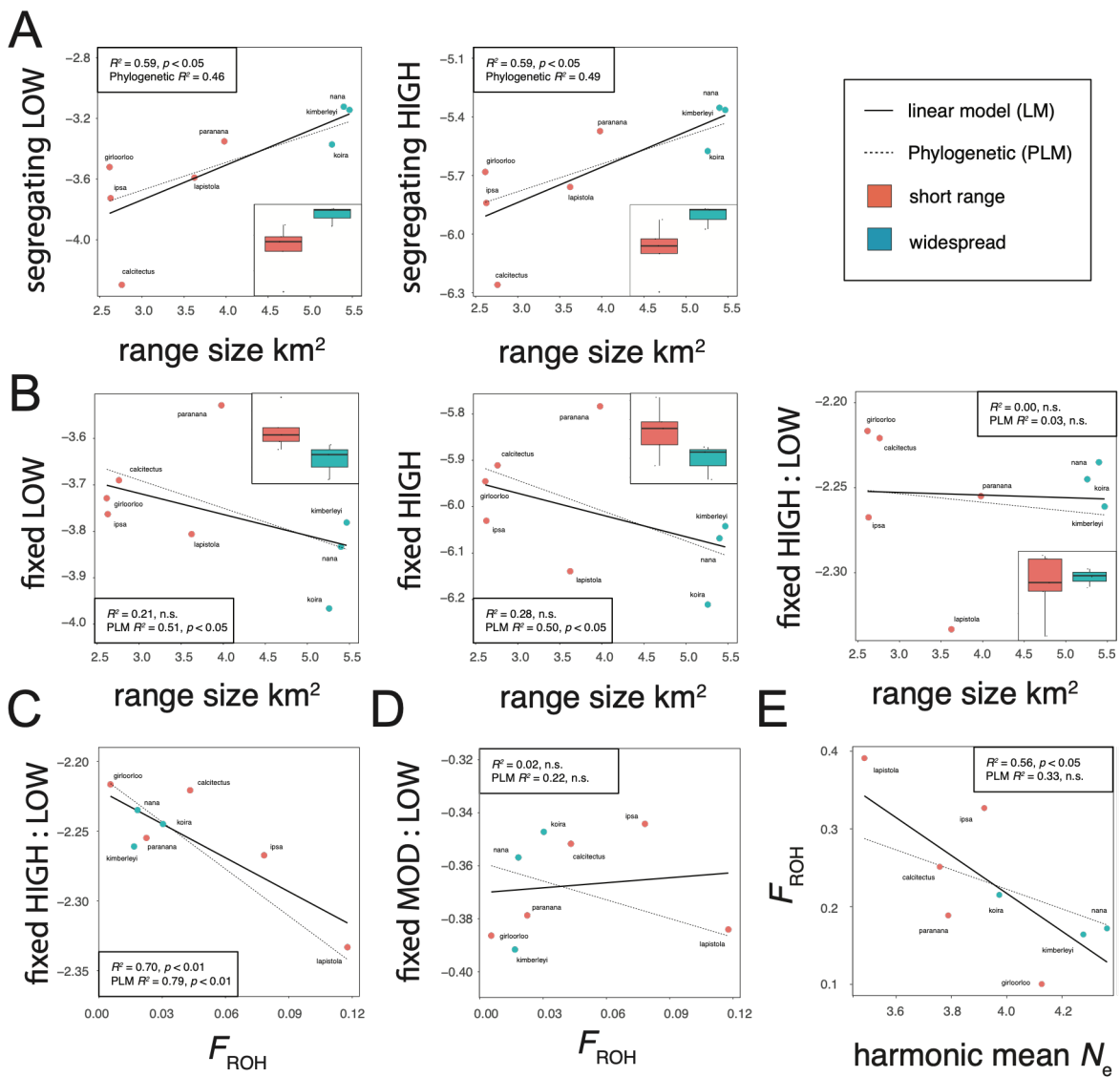


Figure 4. Species with the highest levels of genomic inbreeding show a relative deficit of high-impact deleterious mutations, consistent with genetic purging. Present-day species range size is A) positively correlated with the proportion of segregating mutations in all impact categories ($\log_{10}$ scaled), and B) negatively correlated with the proportion of fixed mutations in all impact categories ($\log_{10}$ scaled), with no excess fixation of high impact compared to low impact mutations in species with present-day small ranges. Moderate impact mutations show the same pattern and are shown in Fig. S8. C) The ratio of high-to-low impact mutations is significantly positively correlated with the proportion of the genome in runs of homozygosity (F_{ROH}), with more inbred species having a lower relative burden of high impact mutations. D) No correlation between the ratio of moderate-to-low impact mutations and F_{ROH} . E) Harmonic mean of past N_e (1k – 100k years before present) is significantly negatively correlated with F_{ROH} in a linear model, but not significant in a phylogenetic linear model. Genetic load and population size measures shown here are log transformed.

No reduced climatic tolerance in short-range endemics

Given intense cyclical dry seasons across parts of the Australian monsoonal tropics, desiccation resistance (as approximated by the rate of evaporative water loss; EWL) is expected to be a crucial adaptive trait in geckos inhabiting this region, and reduced desiccation resistance (i.e. higher EWL rate) may explain why SRE species remain confined to small geographic areas. To investigate this, we analysed previously published data on rates of evaporative water loss (EWL)^{42,43} for the eight focal species in this study. We found few differences among species in rates of EWL overall, and no pervasive difference between SRE and widespread species (Fig. 5). Although ‘species’ had a significant effect on EWL after accounting for surface area

(Table S5), the only statistically significant, within-group pairwise comparison was a lower rate of EWL in the short-range *G. girloorloo* compared to the short-range *G. paranana* ($p = 0.016$; Fig. 5; Table S6). Notably, the lowest and highest mean surface-area (SA)-adjusted rate of EWL both occurred in short-range species of the *nana* group (*G. girloorloo* and *G. paranana*, respectively), and within the *australis* group the highest rate of EWL was in the widespread *G. koiri*. Consistent with this, a phylogenetic ANOVA showed no effect of range type on SA-adjusted EWL (phylANOVA: $F_{1,6} = 0.022$, $p = 0.893$; Table S7). These results suggest that SRE species are not consistently different with respect to desiccation resistance compared to their widespread relatives. Across all species, including both SRE and widespread lineages, previously published data shows that EWL rates were significantly lower under dry-season than wet-season conditions, demonstrating high plasticity for this key physiological trait under climatic extremes⁴³.

359

360 **Discussion**

361 Species trapped in evolutionary refugia, with a long-term history of small population size, are
362 expected to experience genomic erosion¹⁵ and evolve narrow environmental tolerances^{7–11},
363 reducing their capacity to respond to future environmental change. Despite reduced neutral
364 diversity and elevated genomic inbreeding, we find no evidence for an accumulation of
365 deleterious mutations in short-range endemic species, and no evidence for a pervasive
366 reduction in physiological tolerance. This challenges expectations about the intrinsic
367 vulnerability of relictual species to extinction, and demonstrates the power of combining
368 genomic and physiological data to characterise the consequences of long-term persistence in
369 evolutionary refugia. Whole genome data, anchored by new chromosome-level reference
370 genomes for *Gehyra*, have been key in facilitating this. These resources have enabled us to
371 explicitly reconstruct the demographic history of short-range and widespread species, notably
372 revealing a recent demographic contraction in *G. girloorloo*, and to compare runs of
373 homozygosity and genomic proxies of genetic load in short-range endemics and their
374 widespread relatives.

375

376 We found a significantly negative relationship between genomic inbreeding (as measured by
377 F_{ROH}), and the ratio of high-to-low impact, fixed mutations, meaning there is a relative deficit
378 of high-impact, putatively harmful mutations in the most inbred species. This indicates that
379 deleterious mutations have been removed via genetic purging in species with a history of
380 inbreeding, by exposing harmful mutations to selection in the homozygous state. For example,
381 the short-range endemic *G. lapistola* had the highest inbreeding (Fig. 2E), the lowest long-term
382 N_e (Fig. 3), and the lowest ratio of high-to-low impact mutations (Fig. 4C). These results
383 indicate that despite sustained low effective population size and high inbreeding, selection has

still been strong enough to act on deleterious alleles with the highest predicted fitness impact (i.e. loss-of-function mutations, including frame shift or stop codon mutations). This pattern was not as strong for moderate impact (i.e. missense mutations) mutations (Fig. 4D), suggesting that the negative fitness consequences of moderate impact mutations may not be significant enough to overcome the strength of genetic drift in very small populations. This result is consistent with nearly neutral theory⁴⁴, where small N_e weakens the efficacy of purifying selection against slightly deleterious mutations. Similar patterns have been observed in the extinction of mammoths on Wrangel Island, where the frequency of moderate impact mutations continued to accumulate through time despite purging of high-impact mutations¹⁶. Increasingly, empirical evidence has also demonstrated that long-term inbreeding in small and stable populations will expose recessive deleterious mutations to selection, allowing them to be removed from the population via genetic purging^{45–49}, and our results extend this by providing evidence for genetic purging in species trapped in localised evolutionary refugia.

Our historical demographic reconstructions provide empirical evidence for the long-term persistence of short-range endemics at small effective population size, but also highlight a case of recent demographic collapse in *G. girloorloo*, which we infer to have undergone a very recent population contraction (Fig. 3). This species is currently restricted to the Devonian limestones of the southern Kimberley, where it co-occurs with *G. calcitectus*, a species with a long-term history of small N_e . This region is an endemism hotspot, harbouring a diversity of relictual, small range lineages⁵⁰. Given similar present-day distributions, we might have expected these species to share similar historical demographies. In contrast, the stark difference we recover here demonstrates the need to characterise the long-term demographic trajectory of species, to in turn understand their intrinsic vulnerability to environmental change. Our demographic reconstructions are robust to the potential confounding effects of population

structure and admixture, due to our intentional sampling design (see Methods), quantification of lack of within-species population structure among our sequenced samples (PCAs in Fig. 2A and 2B), and absence of historical and recent admixture in the sampled populations⁵¹. The recently-collapsed *G. girloorloo* also shows the lowest levels of inbreeding of all species (Fig. 2E), and a relative excess of fixed, high-impact mutations (Fig. 4C). While it is likely that *G. girloorloo* has not reached demographic equilibrium, the excess of high-impact, fixed mutations may reflect the consequences of demographic collapse from a recently large or widespread population to a very small (412 km²) range (Fig. 2). This reinforces the importance of historical demography in determining the dynamics of putatively deleterious mutations^{46,52–54}, and highlights the potential for segregating load to be rapidly converted to realised load in populations undergoing dramatic range collapse.

By integrating results from physiology experiments, our results provide a rare, complementary perspective on the consequences of long-term persistence in climatic refugia. Comparative analyses suggest that small-range species can occur in areas characterised by reduced climatic variability and greater topographic complexity⁵⁵. Slatyer et al.⁵⁶ identified environmental niche breadth as an important predictor of geographic range size, and emphasised the need for comparison of niche breadth across short range and widespread species. This has rarely been done, however, experimental studies of thermal physiological limits in beetles and *Drosophila* have suggested that small range species will have narrower thermal limits, although reduced plasticity is not consistently observed^{8,13,57}. Despite its likely importance, there have been fewer comparative analyses of the hydric niche compared to the many studies of the thermal niche (but see^{58–60}). Given the dramatic transitions between wet and dry seasons across parts of the Australian monsoonal tropics, and annual periods of up to nine months without significant rainfall in southern areas of the Kimberly and Top End, desiccation resistance (as approximated

by rate of evaporative water loss; EWL) in this region is expected to be a crucial adaptive trait. Evidence for reduced desiccation resistance (i.e. higher EWL rates) in short-range endemic species would have provided a potential physiological explanation for why these lineages are confined to evolutionary refugia. In contrast to this, we found no evidence that short-range *Gehyra* have reduced physiological tolerances, in terms of EWL, compared to their widespread relatives (Fig. 5). In a previous set of physiology experiments looking at broader *Gehyra* lineages, all species had lower EWL under dry-season conditions, compared to wet-season conditions⁴³, indicating substantial capacity to respond physiologically to seasonal changes in water availability. Although short-range species in this study have persisted in evolutionary refugia with long-term stable environments, it is important to note that these habitats still exist within the fluctuating monsoonal region of northern Australia, and so their physiological resilience may in part be maintained by their exposure to seasonal variance. This may not be the case for all species in evolutionary refugia, where environments are less seasonal. As is the case for temperature^{61–63}, it is possible that behavioural responses, such as activity time and microhabitat choice, are also important to understanding the hydric niche and its evolution in reptiles^{58,60,64,65}. However, our results here suggest that there is no consistent difference in the intrinsic hydric physiological tolerances of short-range *Gehyra* compared to widespread congeners in our focal species in northern Australia.

Together, our results challenge the assumption that geographic restriction necessarily reflects an intrinsic increase in extinction vulnerability. This does not mean that relictual species are not vulnerable to extinction, as small-range populations remain at high risk via extrinsic threats. Relictual *Gehyra* species may have avoided the potential proximate negative consequences of small population size, including mutational meltdown and subsequent extinction vortex, and have comparable intrinsic and plastic physiological responses, in terms of evaporative water

loss, to their widespread counterparts. However, their restricted distributions make them susceptible to stochastic or catastrophic events that affect their entire range, including habitat destruction, anthropogenic climate change, and increasing natural disasters. In addition, reduced genetic diversity and high inbreeding in short-range species may also limit their capacity to respond to future environmental change^{66–69}, although genetic diversity may not predict short-term adaptive potential⁷⁰. We highlight that even when their contemporary distributions make them appear highly threatened, not all short-range species share the same intrinsic vulnerabilities. For example, our results indicate that the intrinsic vulnerability of recently contracted populations (e.g. *G. girloorloo*) to extinction via mutational meltdown may be higher than that of species with long-term small N_e . In recently contracted populations, intrinsic vulnerabilities may exacerbate the extrinsic vulnerability of extinction via catastrophic environmental events in ways that do not apply to species with long-term small N_e .

More broadly, our results show that present-day geographic restriction alone provides an incomplete picture of extinction risk. Understanding the processes that generate and maintain small ranges is essential for distinguishing the relative contributions of intrinsic and extrinsic threatening processes to extinction risk. Disentangling these factors is increasingly crucial to implementing effective conservation prioritisation and management of threatened species in a rapidly changing world.

Methods

1. Reference genomes

1.1 Sampling and sequencing

To generate high-quality reference genomes to anchor our analyses across the *Gehyra* radiation, we collected tail, liver and blood samples from a female *Gehyra lapistola* (*australis* group) and female *Gehyra paranana* (*nana* group) from Litchfield National Park (sample details in Table S1), under ethics permit ANU A2022_07. Females were chosen for reference genomes, as previous research suggests ZW sex determination in some *Gehyra* lineages^{38 71,72}. Tissue samples were flash frozen at -80°C immediately after collection to preserve high-molecular weight DNA. We extracted high-molecular weight DNA from tail tissue using either a magnetic bead-based protocol⁷³ (*G. lapistola*) or a Circulomics NanoBind high molecular weight extraction kit (*G. paranana*). Extractions were enriched for high-molecular weight fragments using a Circulomics Short-Read Eliminator Kit (15kb, *G. lapistola*) or a BluePippin (20kb, *G. paranana*). Resultant high-molecular weight libraries were prepared for Oxford Nanopore sequencing using a Ligation Sequencing Kit (*G. lapistola*; SQK-LSK109, *G. paranana*; LSK114). Each library was sequenced using a promethION flow cell at the Biomolecular Resource Facility at the Australian National University, as well on two minION flow cells. Base calling was performed with Guppy 6.2.11 using a high accuracy model. Using the remaining extracted DNA for each sample, we separately prepared Illumina libraries for short-read polishing using an Illumina Tagmentation DNA Prep Kit, sequenced on one lane of an Illumina NovaSeq 6000 S1 (*G. lapistola*) or NovaSeq 6000 S2 (*G. paranana*) flowcell.

1.2 De-novo genome assembly, contaminant removal and polishing

We trimmed sequencing adapters from long-read data using NanoLyse and removed reads <1kbp and <7 quality score using NanoFilt⁷⁴. Using filtered reads, we performed *de novo*

assembly using Flye 2.9⁷⁵ with three long-read polishing iterations. To identify potential sequencing contaminants from these initial assemblies, we used blobtools⁷⁶ to find outlier contigs based on both sequencing coverage and hits to the BLAST nucleotide database. In the initial *G. lapistola* assembly, we identified 16 very small (< 0.2 Mb), low coverage contigs originating from phyla Arthropoda and Streptophyta, and in the *G. paranana* genome, three contigs from phylum Arthropoda (< 0.05 Mb). These contigs were classified as contaminants and removed from the assemblies. Using Purge Haplotigs⁷⁷, we checked and found no evidence for any separately assembled haplotypes (i.e., haplotigs) in each draft assembly (Fig. S1). These assemblies were based on filtered, long-reads of 36X average genome-wide coverage for *G. lapistola* and 48X for *G. paranana*.

To further error-correct our long-read assemblies, as recommended by⁷⁸, we performed two additional rounds of long-read polishing using Racon 1.5.0⁷⁹, followed by two rounds of short-read polishing using NextPolish 1.4.1⁸⁰ and three rounds of short-read polishing using Haplo-G⁸¹. To retain only nuclear contigs in each assembly, we used MUMmer⁸² to identify and purge contigs matching to the *Gekko gecko* whole mitochondrial genome (NC_007627.1), and filtered contigs below 1000bp length from the draft assembly.

1.3 Hi-C scaffolding, annotation and synteny analysis

For chromosome-level scaffolding, we used flash-frozen tail tissue from *G. lapistola* and *G. paranana* to prepare chromosome conformation capture libraries using a Proximo Hi-C Kit (Phase Genomics), following the manufacturer's protocol. We pooled and sequenced both uniquely dual-indexed Hi-C libraries together on one lane of an Illumina NovaSeq 6000 SP flowcell. We compared two different approaches to scaffolding. Hi-C reads were first aligned to each draft assembly using the Juicer pipeline⁸³, and then either the 3D-DNA⁸⁴ or YaHS⁸⁵

pipeline was used to scaffold contigs in the draft assembly and generate contact maps. In both cases, Juicebox Assembly Tools was then used to correct any assembly errors in the final assembly (Fig. S2). We compared contiguity and BUSCO completeness of both approaches, and found that the 3D-DNA scaffolded assembly was more complete for *G. lapistola*, while the YaHS scaffolded assembly was more complete for *G. paranana*. As such, these respective final scaffolded assemblies were retained for downstream analysis. The final scaffolded genomes were then annotated using previously published *Gehyra* transcriptomic data (SRR3901730), via NCBI's Eukaryotic Genome Annotation Pipeline (<https://github.com/ncbi/egapx>). To examine synteny in our two new *Gehyra* reference genomes, we used ChromSyn v1.3.0⁸⁶ to generate a chromosome-level synteny plot. As we were interested in chromosome-scale synteny, we fed the primary chromosome-level scaffolds in this analysis to BUSCO v5.0 using the 'tetrapoda_odb10' database to identify orthologous regions, ran diploidocus⁸⁷ and TIDK to predict telomeric regions, and plotted the results with ChromSyn in R.

1.4 Identifying a putative sex-linked scaffold

Given the diversity of sex-determination systems in geckos^{88,89}, and the potential for sex-linked chromosomes to display a distinct evolutionary history compared to the rest of the genome, we tested for the presence of a differentiated sex-chromosome in our two new *Gehyra* genome assemblies. First, we leveraged short-read, whole-genome data from males and females of eight *Gehyra* species (as part of the whole genome resequencing data described below), to investigate the distribution of heterozygous sites across assembled scaffolds in our two reference genomes. Scaffold-specific elevated heterozygosity in individuals of the heterogametic sex (expected to be the females in *Gehyra*) may be evidence of unphased, partially differentiated sex-chromosomes. This is because the sequences of the Z and W

chromosomes can be similar enough that they will map to the same reference scaffold, but have sufficient sequence divergence that they elevate heterozygosity. We took one male and one female from each species and counted the number of heterozygous sites in 1kb non-overlapping windows, averaging these counts in sliding windows of 1 Mb across the genome. We plotted these values along each scaffold to generate a sliding window, genome-wide visual summary of heterozygosity.

Based on the above, we identified scaffold 5 in the *G. lapistola* genome assembly, and in all three other species in the *australis* group, as a putative sex-chromosome. There was no evidence, based on heterozygosity, of a differentiated sex chromosome in the *G. paranana* genome, nor any of the other three species in the *nana* group. To further validate this contig as a putatively sex-linked chromosome in the *australis* group, we leveraged our previously published dataset of reduced representation data (DARTseq) from across the broader *australis* group radiation of individuals of known sex³³, and applied the sex-linked function⁹⁰ in *dartRverse* in R to test for sex-linked loci on scaffold 5.

2. Whole-genome resequencing and analysis

2.1 Sampling, DNA library preparation and sequencing

We targeted eight species, spanning phylogenetically related comparisons within the Australian *Gehyra* radiation of short-range endemics (SRE) and their widespread relatives. This included four closely related species in the *australis* group: *Gehyra koiri* (widespread, n = 11), *G. lapistola* (SRE, n = 12), *G. calcitectus* (SRE, n = 10), and *G. ipsa* (SRE, n = 9), and four species in the *nana* group; *G. nana* (widespread, n = 11), *G. paranana* (SRE, n = 10), *G. kimberleyi* (widespread, n = 8), and *G. girloorloo* (SRE, n = 11). Here, we used the definition of a short-range endemic species having a total distribution of <10,000 km² (see¹⁸). Prior

studies using genome-wide SNPs from reduced-representation data have confirmed little or no historical admixture between species, even where there is mtDNA introgression^{33,51}. Our sampling was designed to ensure sequenced individuals originated from panmictic populations, and to avoid sampling within-species geographic population structure that may bias demographic inference. Guided by previous studies of population structure in *Gehyra*^{28,29,33,91}, sampling for each species was restricted to a single, largely contiguous rocky area where individuals belonged to the same subpopulation. The exception was the widespread *G. kimberleyi*, which is a habitat generalist, and for which sampling covered a larger area across the southern and south-western Kimberley (again, within a single subpopulation). Details and collection localities of all 82 samples used for whole-genome, short-read sequencing are provided in Table S1.

Genomic DNA was extracted using a high-salt extraction protocol⁹², and then quantified using a Qubit fluorometer and visualised on an agarose gel to determine the distribution of fragment sizes. Extracted DNA was normalised to ~14 ng/μL in 110μL and sonicated using a Bioruptor NGS (Diagenode, Inc), followed by size-selection using a double-ended bead clean (Sera-Mag SpeedBeads) to retain fragments between 200-600 bp. We performed DNA library preparation following a modified NextFlex Rapid DNA-Seq Kit 2.0 protocol (Revvity), using half volumes for all reactions (192 samples prepared per kit). Samples were uniquely dual-indexed, and amplified with two independent PCR reactions of 8-9 cycles (initial denaturation: 98°C for 30s; repeated 3-step cycle: 98°C for 15s, 65°C for 30s, 72°C for 30s; final elongation: 72°C for 2 min). Final, dual-indexed, amplified libraries were pooled across two Illumina NovaSeq 6000 S4 flow cells (300 cycles) and sequenced at the Biomolecular Resource Facility at the Australian National University.

2.2 Bioinformatic processing and variant calling

Raw sequence reads were trimmed using fastp⁹³ to remove adapter sequences and low-quality reads (minimum length = 30, with base correction for paired-end reads enabled). Trimmed reads were mapped to either the *G. lapistola* (for *australis* group species) or *G. paranana* (for *nana* group species) reference genomes using NextGenMap⁹⁴, with the sensitivity parameter estimated from the input reads to enable dynamic estimation of the divergence of each species from the reference genome. We removed PCR and optical duplicates from the resultant SAM files using samremovedup.py (github.com/pontusssk/samremovedup). We then fed deduplicated BAM files into DeepVariant (version 1.5.0 GPU) for variant calling, using a multi-tiered, cohort-genotyping approach. Variant calling was first performed on a sample-by-sample basis, and then gVCF output files were fed to GLnexus (version 1.4.1) for joint variant calling using two different groupings; 1) clade-level cohorts, i.e., *australis* group or *nana* group, to perform combined principal component analysis, and 2) species-level cohorts, for all other downstream analyses. This cohort-genotyping approach generates integrated, consistent multi-sample VCFs at both the clade- and species-level.

2.3 Variant filtering and exploratory principal components analysis

We first used vcftools to remove the putative sex chromosome from all variant files that included *australis* group species (scaffold 5 in the nomenclature of the reference genome assembly of *G. lapistola*), to avoid spurious inferences that can result from co-analysing genomic regions with contrasting evolutionary and demographic histories. No sex-linked contigs were identified in the *nana* group clade.

We then filtered cohort-called, multi-sample VCF files with the following sequential filtering steps⁹⁵: (1) minor allele count ≥ 2 , (2) quality value ≥ 20 , (3) minimum mean depth ≥ 10 , (4)

minimum depth ≥ 3 , (5) missing sites $< 20\%$ across individuals, (6) remove indels, (7) only keep samples with $\leq 50\%$ individual missingness, (8) sites with missing data $< 5\%$ and (9) minor allele count ≥ 2 . Using the final filtered clade-level joint-called SNP dataset, we performed principal component analysis (PCA) in SNPrelate (Zheng et al., 2012) and gdsfmt (Zheng et al., 2017) using our final filtered SNP dataset, to confirm no samples were misidentified, and confirm the absence of undetected population structure within species that could impact downstream analyses.

2.4 Genomic diversity and inbreeding

We first used the single-sample gVCF files, generated prior to cohort genotyping, to estimate unbiased individual heterozygosity, as these files include both variant and invariant sites. We used bcftools v1.10.2 “convert” to expand compressed invariant sites records, and only considered sites with a minimum genotype quality of 20, a minimum read depth of 5X and removed indels. Using filtered, single-sample gVCF files containing both variant and invariant sites, we calculated individual heterozygosity as the proportion of autosomal heterozygous sites out of total valid autosomal sites per individual. This approach allows us to consider all observed, callable sites for each sample – providing an unbiased estimate of individual autosomal heterozygosity^{33,96}. We then used our filtered, species-level cohort VCFs in vcftools to estimate autosomal nucleotide diversity (π) in sliding windows of 10 kb for each population, and the inbreeding coefficient (F) and pairwise relatedness⁹⁷ for all samples within each species.

In addition to traditional inbreeding metrics, the number and length of runs of homozygosity (ROH, i.e., segments of the genome with little to no heterozygosity) can both estimate individual ROH-based inbreeding coefficients (F_{ROH}) and provide information about how

recent inbreeding is. Using PLINK v1.9⁹⁸, we identified ROH using a sliding window size of 50 SNPs (*homozyg-window-snp 50*), allowing no more than 1 heterozygous site per homozygous window (*homozyg-window-het 1*), and defined sites with only one callable allele as missing (*--vcf-half-call missing*). The ROH-based inbreeding coefficient F_{ROH} was estimated as the total proportion of the autosomal genome that is contained within ROH. Comparisons between paired SRE and widespread species were performed using a Welch's two-sided *t.test* in R. To investigate the relationship between heterozygosity, F_{ROH} and present-day range size, we performed ordinary least-squares linear regression and phylogenetic generalised least-squares regression (to account for phylogenetic non-independence of species), implemented in the R package *phylolm*⁹⁹.

2.5 Reconstructing demographic history

For demographic analyses, we started with individual VCF files following variant calling with DeepVariant, and only considered sites with genotype quality > 20, a minimum read depth >0.5X that of the mean coverage, a maximum read depth <2X the mean coverage, and excluded indels. We used *msmc-tools* (*vcfAllSiteParser.py*) to create mask files in BED format, and then used *generate_multihetsep.py* to combine the individual masked VCFs into species-level input files, and ran MSMC2 with an atomic time interval of $-p 1*2+25*1+1*2*3$ and 20 iterations. To visualise demographic history reconstructions, we used mutation rate estimates based on parent-offspring trios of the Texas banded gecko⁴¹, a species in the family Gekkonidae and the closest relative to *Gehyra* for which such estimates are available. Bergeron et al.⁴¹ present two alternate mutation rates for the Texas banded gecko; 3.17E-09 and 1.96E-08 per site per year, with the latter rate adjusted to account for variance in parental age. To capture the potential variance in mutation rate for geckos, we considered both mutation rates, multiplied by a generation length of 2.5 years (based on estimates from *G. variegata*⁴⁰) to obtain two alternate

per-generation mutation rates for *Gehyra* (7.93e-09 and 4.89e-08 per site per generation, respectively). We plotted our results using both alternative rates for comparison, and following⁴¹, we present the modelled rate in the main text results.

2.6 Quantifying putatively harmful mutations

To investigate the burden of harmful mutations in SRE compared to widespread species, we first remapped our cleaned sequence reads to the opposite reference genome from the clade in which they originated from (i.e., *australis* group samples were mapped to *G. paranana*, and *nana* group samples were mapped to *G. lapistola*). This was done to polarise putatively deleterious sites against an ‘outgroup’ of consistent phylogenetic distance. All bioinformatic and filtering steps of these ‘cross-mapped’ files were carried out as described in the above sections, with regions mapped to the putative sex-chromosome removed prior to analysis (i.e., for the *australis* group samples now mapped to the *paranana* genome, this is scaffold 2 and 14 in the *paranana* co-ordinate system). Then using SnpEff (version 5.2f¹⁰⁰), we built a database for both the *G. lapistola* and *G. paranana* reference genomes, using genome annotations described in section 1.3 above. For each VCF file, we annotated variants as either LOW, MODERATE, or HIGH impact. Based on the annotation system in SnpEff¹⁰⁰, HIGH impact variants are assumed to have a disruptive impact on protein function, potentially causing truncation or loss-of-function (e.g. premature stop codons), MODERATE impact sites are missense mutations or in-frame insertions/deletions, that might alter protein effectiveness, and LOW impact sites are assumed to be mostly neutral or unlikely to alter protein behaviour (e.g. synonymous variant). For population-level analyses, we then used bcftools to categorise variants as either segregating (by excluding sites for which all individuals had the same homozygous genotype – either 0/0 or 1/1), or fixed (by retaining only sites that were entirely in the alternate homozygous state ‘1/1’ across all individuals within the population). As we

were interested specifically in the accumulation of mutations within each species, rather than mutations that arose in ancestors and subsequently became fixed across multiple species, we excluded alternate homozygous variants that were fixed in all individuals of two or more species. We then quantified the relative mutational load for each impact category as $\frac{\text{number sites of each impact}}{\text{invariants} + \text{variants}}$, and also separated out the fixed and segregating load proportion within each category for each population, as well as ratios of HIGH to LOW impact, and HIGH+MODERATE to LOW impact mutations. To investigate how genetic load accumulates in populations with contrasting near- and long-term demographic histories, we investigated whether current range size, mean F_{ROH} , and the harmonic mean of N_e (calculated for two different windows; between 1k – 10k years before present, and 1k – 100k years before present), taken from MSMC2 demographic reconstructions (see above), predicted either segregating load, fixed load, or their ratios, across all impact categories. To test these correlations, we used both an ordinary least-squares linear regression and phylogenetic generalised least-squares regression to account for phylogenetic non-independence of species, implemented in the R package *phylolm*⁹⁹.

3. Comparative physiology

To test the hypothesis that SREs have evolved narrower physiological tolerances as a result of long-term persistence in evolutionary refugia, we re-analysed previously published estimates of evaporative water loss (EWL) rates from animals measured in the field during the early dry season⁴². In brief, whole-gecko EWL rates were measured using an open-flow system with constant temperature and flow of dehydrated air with the animal at rest. We subset the data to include only our eight focal *Gehyra* species and performed statistical analyses in R v4.4.2, with surface area (SA) included as a covariate in analyses to account for the scaling of EWL with body size. We modelled log(EWL) as a function of SA and species using a linear model (*lm*

function), having first confirmed that the slope of log(EWL) on SA did not differ among species. We then used the *emmeans* package to estimate SA-adjusted mean EWL for each species at the overall mean SA (33.48 cm²) and to compare all species pairs with Tukey-adjusted post hoc tests. To test whether SA-adjusted EWL differed between widespread species and short-range endemics while accounting for shared ancestry, we performed a phylogenetic ANOVA (*phylANOVA* function in *phytools* v2.5-2¹⁰¹) on the SA-adjusted species means, specifying a Brownian motion model of trait evolution (10,000 iterations). We used a previously published species tree phylogeny based on genome-wide exon data³³, with the tree subset to include only the eight relevant taxa included here.

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764

765 **Data Accessibility Statement:** Raw sequencing reads have been deposited in the NCBI
766 Sequence Read Archive under BioProject PRJNA1540017, and reference genome assemblies
767 and processed variant call files have been deposited in Figshare. Both the NBCI short-read
768 BioProject and Figshare repositories will be made public upon manuscript acceptance.

769

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771 processed and analysed the population genomic data. ER and RS performed laboratory work.
772 SMZ, KC and CM performed the fieldwork and sampling. SMZ analysed the physiology
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774

775 **Competing interests:** The authors declare that they have no competing interests.

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Supplementary Figures and Tables

Roycroft *et al.* ‘Genomic costs without functional consequences in short-range endemics trapped in evolutionary refugia’

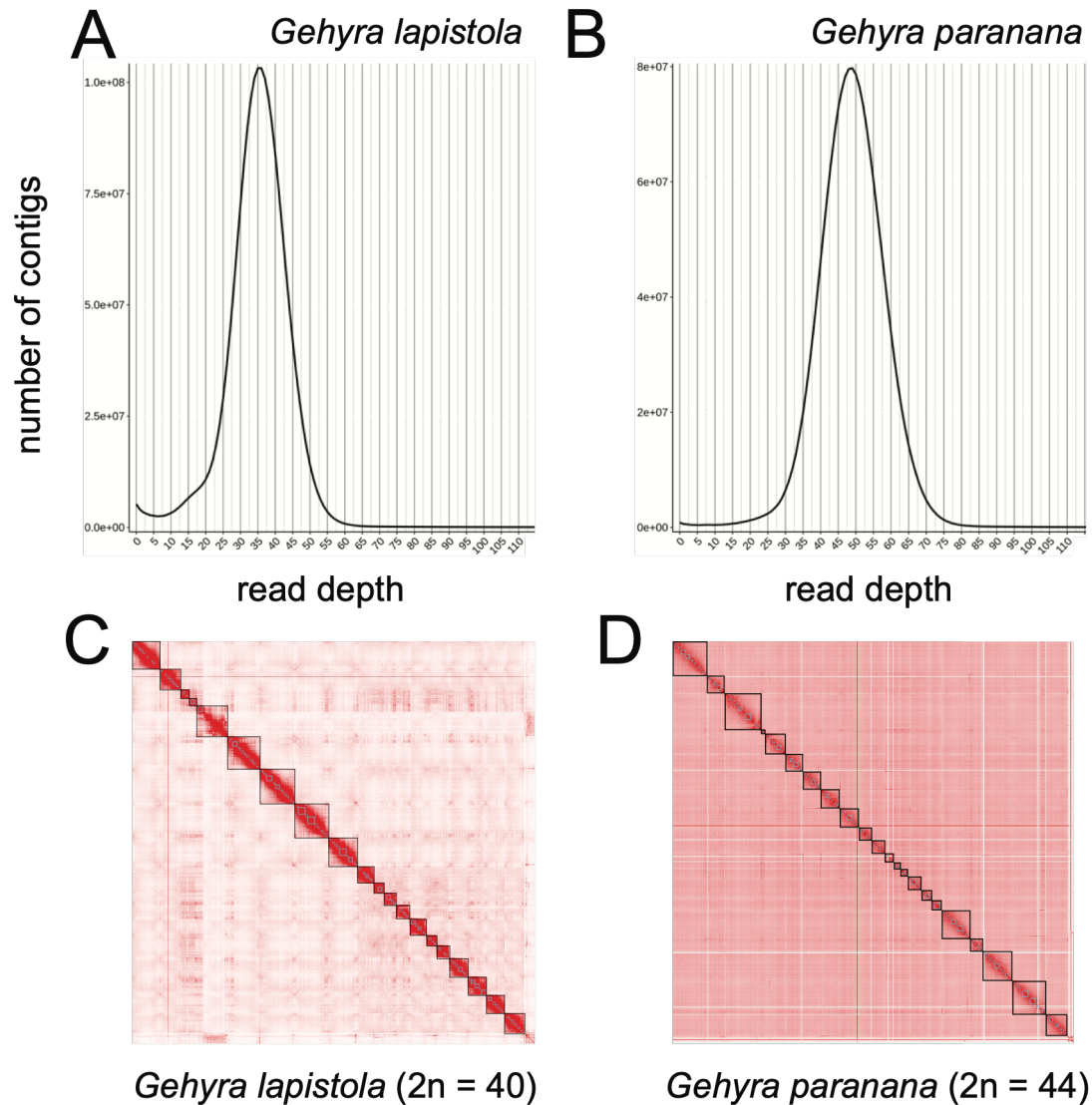


Figure S1. Whole genome contig coverage and contact maps. Related to Figure 1. A) Histograms of read-depth for the *Gehyra lapistola* and B) *Gehyra paranana* long-read assemblies, showing the absence of haplotigs (which would be indicated by bimodal read-depth). C) Hi-C contact maps showing 20 primary scaffolds assembled for *Gehyra lapistola*, and D) 22 primary scaffolds assembled for *Gehyra paranana*. In the *G. lapistola* contact map, scaffold 5 in shows long-range interactions with unplaced contigs (bottom right corner). We

interpret this as evidence that scaffold 5 represents a partially collapsed, partially differentiated neo-sex chromosome in this female-based assembly, i.e. a W chromosome containing partially collapsed ZW segments, with those collapsed Z-segments showing long-range associations with unplaced Z-origin contigs. Related to Figure 1.

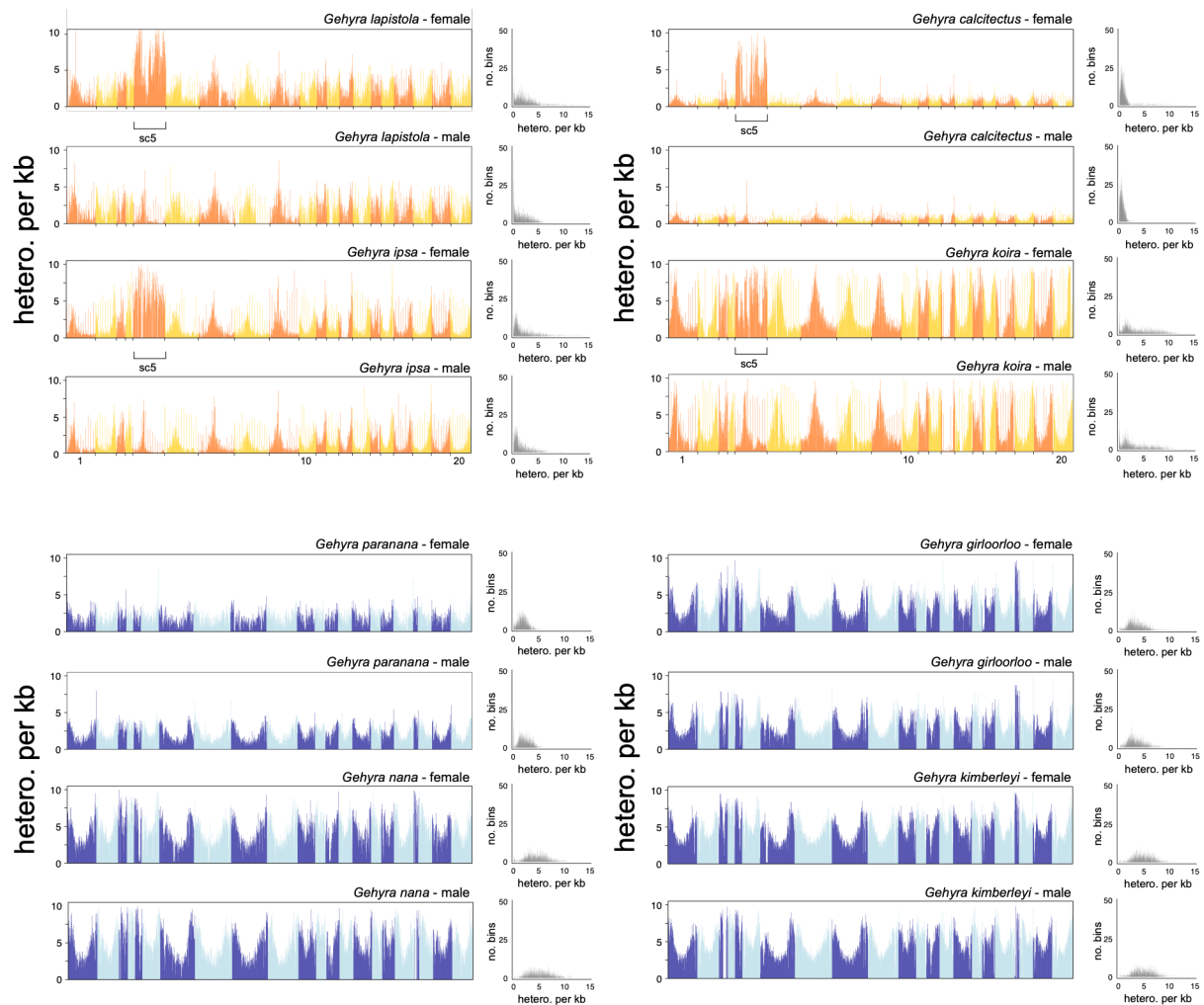


Figure S2. Genome-wide heterozygosity of male and female *Gehyra*, showing elevated female heterozygosity at scaffold 5 in the *australis* group. Related to Figure 1. Heterozygous sites per kilobase-pair window are shown for female (top of each pair) and male (bottom of each pair) individuals from four species in the *australis* group (orange and yellow) and four species in the *nana* group (light and dark blue). Windows are ordered by chromosome-level scaffolds from left to right, with chromosome boundaries indicated by alternating dark and light colours. A grey histogram alongside each plot summarises the distribution of genome-wide heterozygosity. All females of the four *australis* group species shown here show elevated heterozygosity on scaffold 5 (indicated with square brackets), while males do not, which we interpret as resulting from reads from both the Z and W chromosome of females mapping to scaffold 5 (i.e. sex chromosome) in the *australis* group. There is no difference between the heterozygosity of males and females at any contig in the four *nana* group species, with suggesting the lack of a differentiated sex chromosome in these species.

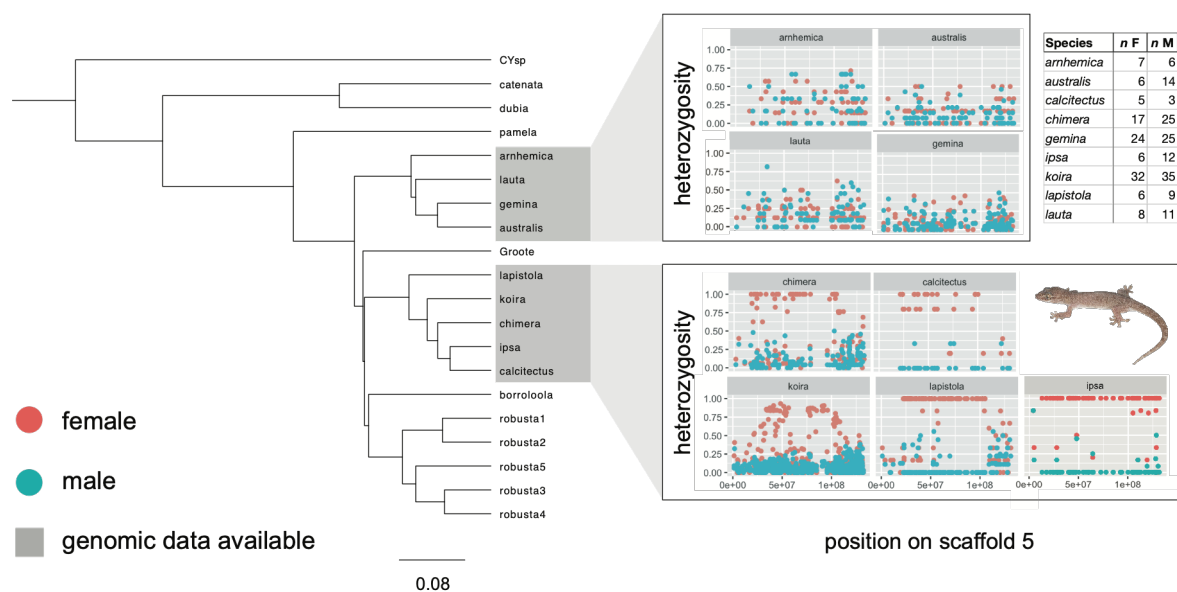


Figure S3. Evidence for a putative novel sex chromosome in a sub-clade of the *australis* group. Related to Figure 1. Phylogenetic tree of the *australis* group, with inset showing heterozygosity of DArTseq data from nine species (Lau et al. 2025) mapped to scaffold 5 of the *G. lapistola* genome assembly. Five species within the *lapistola*-related subclade of the *australis* group (including the four species from which we have whole genome resequencing data, shown in Fig. S2) show evidence for elevated heterozygosity in females (red) compared to males (blue), indicating that scaffold 5 is a putative sex chromosome in these species, originating no later than the crown-age of this clade. In contrast, four species in the *arnhemica*-related subclade of the *australis* group do not show evidence for a differentiated scaffold 5 in females. This, along with the lack of a sex chromosome in four species within the *nana* group (Fig. S2), suggest that this sex chromosome arose after the branching of the *arnhemica*-related subclade.

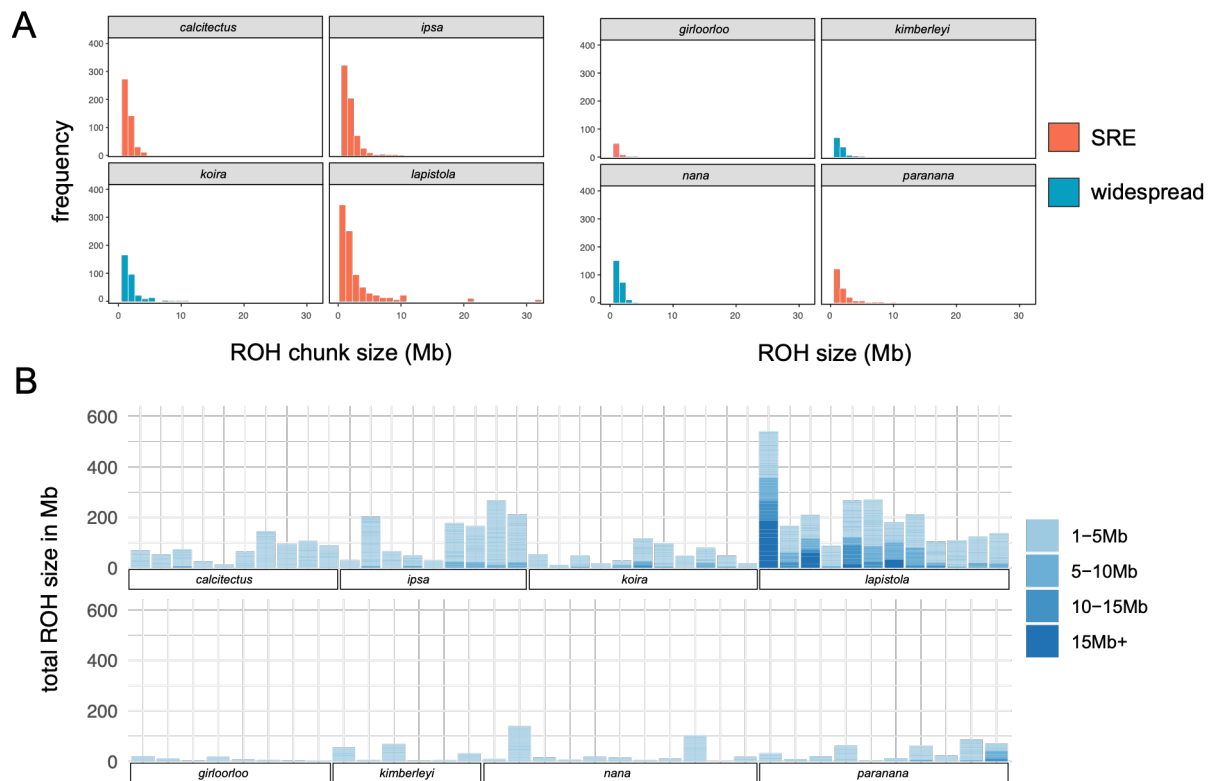


Figure S4. Size distribution of runs of homozygosity (ROH) across eight *Gehyra* species. Related to Figure 2E. A) The frequency of variable ROH chunk size in megabase pairs (Mb) for each species, and B) the total ROH size in Mb shown for individuals, grouped by species. In panel B, larger ROH chunks are shown in darker blue shading. Individual samples that are not shown in panel B had no ROH chunks of at least 1Mb detected (i.e. two *kimberleyi* and three *girloorloo* samples).

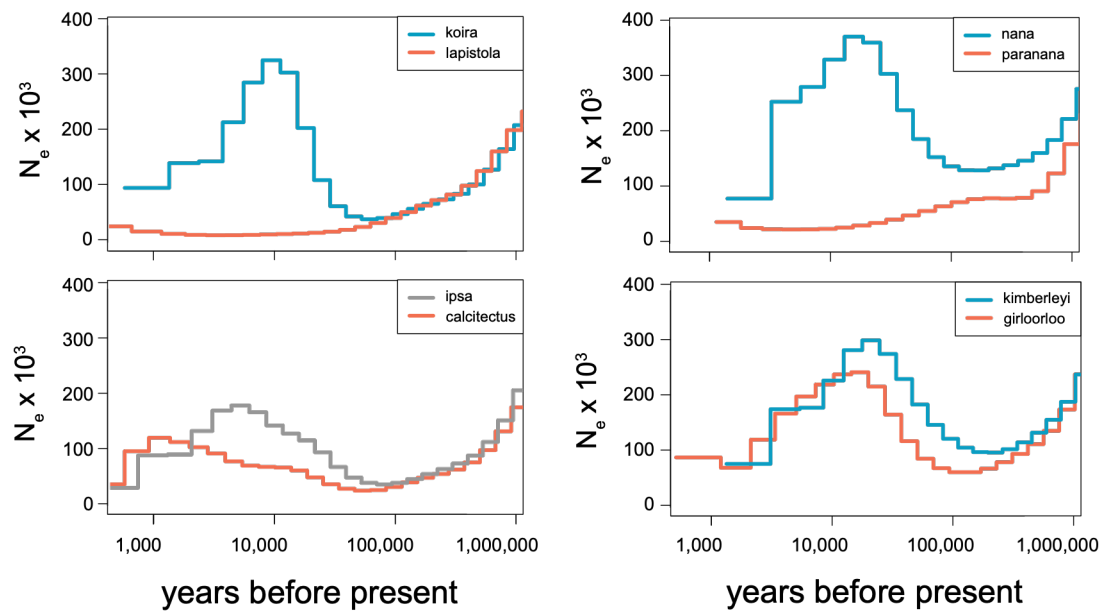
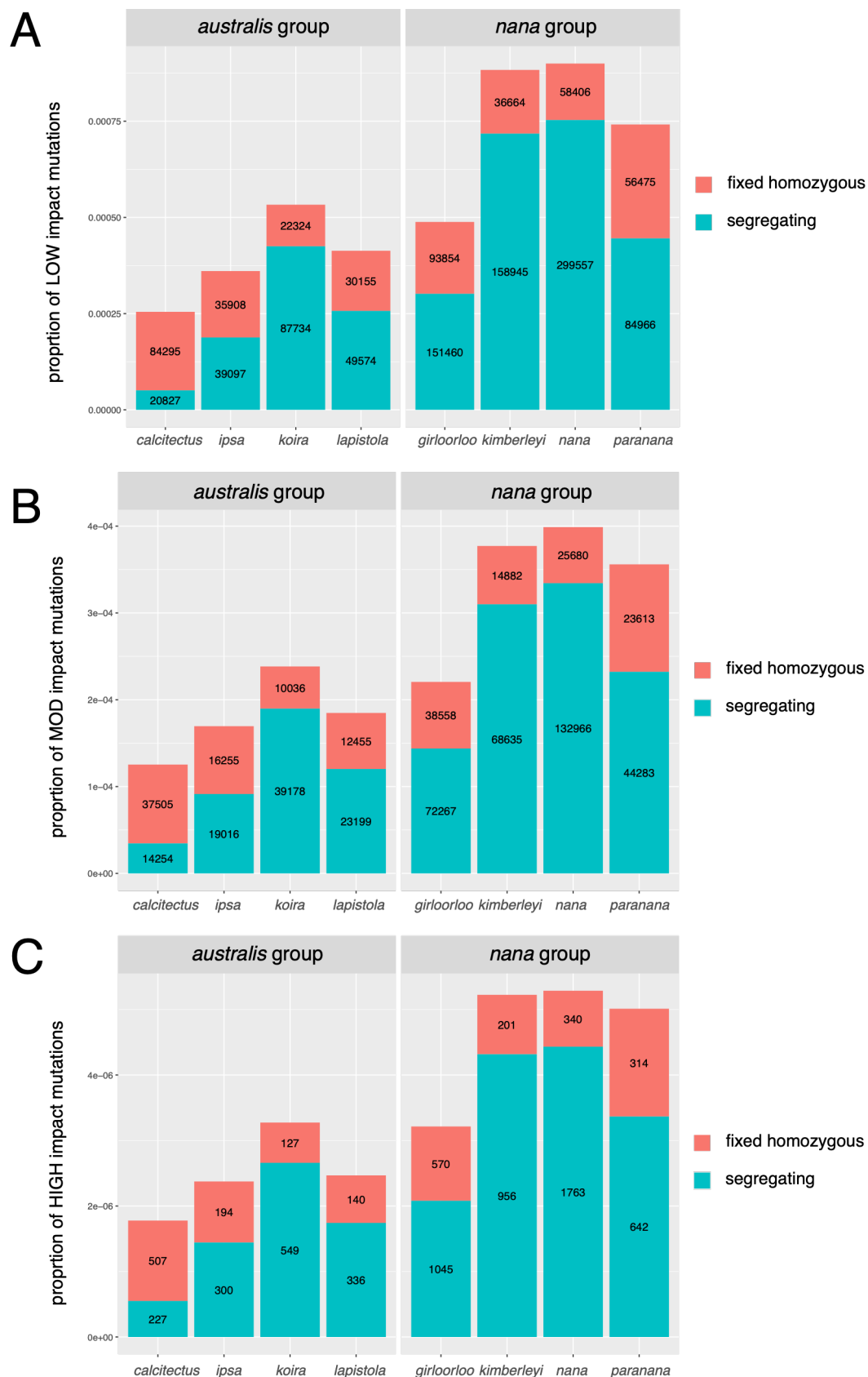


Figure S5. Demographic history reconstruction using MSMC2. Related to Figure 3. Here, reconstructions are based on alternative mutation rate of 3.17E-09 per site per year, and assuming a generation length of 2.5 years.



1117

1118 **Figure S6. Proportion of sites annotated by SnpEff in each functional category. Related**
 1119 **to Figure 4. A) Low impact, B) moderate impact, and C) high impact. For each species, bars**

1120 are further broken down to indicate sites with segregating variation within each population
1121 (blue), and species-specific alternative alleles that were fixed in the homozygous state across
1122 all individuals in each population (red), with counts of each of these categories shown as
1123 numbers.

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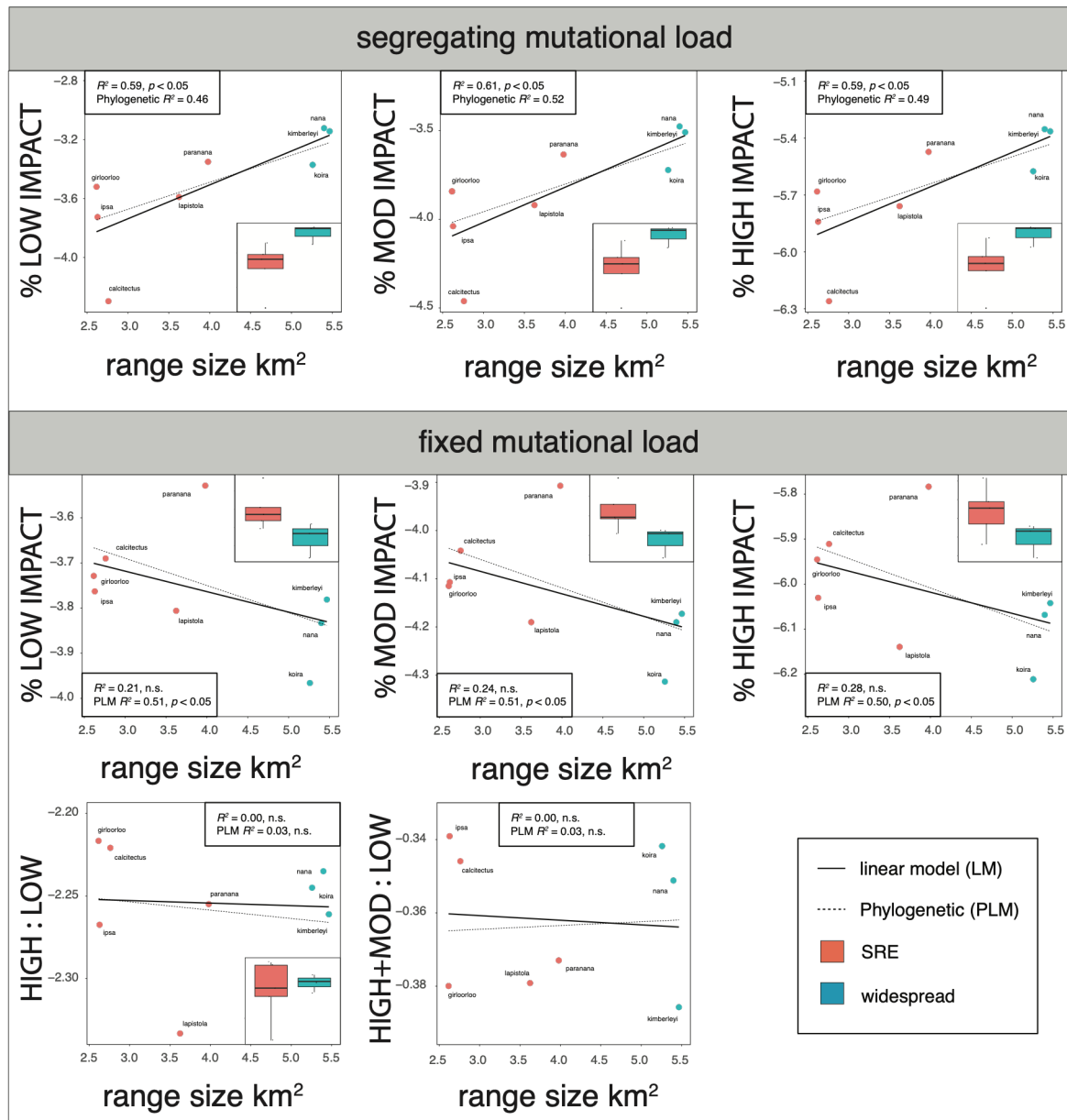


Figure S7. Low, moderate and high impact segregating sites are significantly positively correlated with range size, while fixed sites negatively correlated with range size. Related to Figure 4. There is no correlation between range size and the ratio of high to low impact sites, or high and moderate to low impact sites. All variables shown here are log transformed.

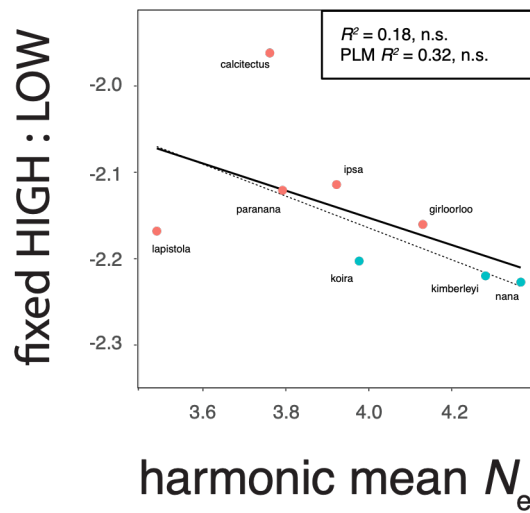
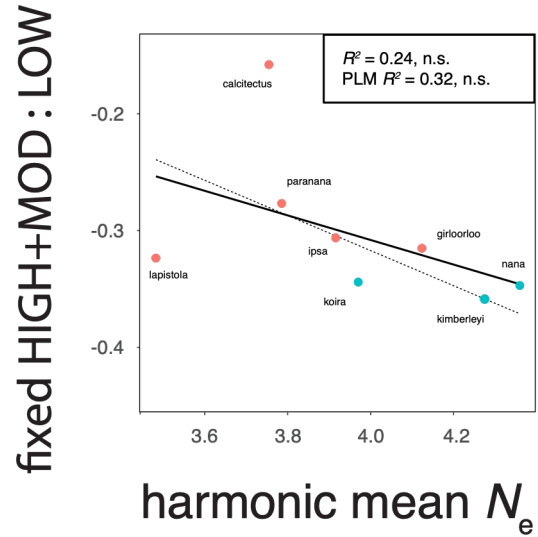


Figure S8. The harmonic mean of N_e is not significantly correlated with genetic load. **Related to Figure 4.** The harmonic mean of N_e (1 – 100k years before present) is taken from MSMC2 reconstructions, is not significantly correlated with the ratio of HIGH to LOW impact fixed load, or HIGH + MODERATE to LOW impact fixed low. The trends shown here and their statistical significance are also consistent when the harmonic mean of N_e was calculated between 1 – 10k years before present. All variables here are shown log transformed.

Table S1. Sample metadata, collection localities, sequencing coverage, and detail on samples included in each analysis

See associated Excel file

Table S2. Reference genome assembly statistics for *Gehyra lapistola* and *Gehyra paranana*. Related to Figure 1. ‘Contigs’ are those assembled in the *de novo* draft assembly from Oxford Nanopore (ONT) reads following all filtering and polishing iterations. ‘Scaffolds’ refer to contigs joined together following Hi-C scaffolding.

	<i>Gehyra lapistola</i>	<i>Gehyra paranana</i>
Assembly size	1.89 Gb	1.73 Gb
Number of scaffolds	6098	1851
Longest scaffold	163751179	156970225
Number of scaffolds > 10Mb	20	22
N50 scaffold length	95544768	87459471
L50 scaffold count	7	7
Genome in scaffolded contigs (%)	97.5%	99%
Genome in scaffolded contigs > 10Mb (%)	97.41%	98.53%
Number of contigs	8738	2572
Contigs in scaffolds	2668	760
N50 contig length	6626880	8794984
Mean coverage of filtered ONT reads	36 X	48 X
Mean coverage of filtered short-read polishing Illumina reads	55X	88X
Complete vertebrate BUSCOs (%)	97.6%	97.4%
Complete tetrapod BUSCOs (%)	96.4%	96.6%

Table S3. Total number of SNPs per cohort/species pre- and post-filtering, and as used for each analysis. Related to Figure 2. # seg. sites = the number of segregating sites in genetic load analyses, # unique alt homo. sites = the number of alternative homozygous sites unique to each species used in genetic load analyses.

<i>Analysis</i>	<i>Cohort</i>	<i># filtered SNPs</i>	<i># unfiltered SNPs</i>	<i># seg. sites</i>	<i># unique alt homo. sites</i>
<i>PCA</i>	<i>australis</i>	11150501	67158423		
	<i>group</i>				
	<i>calcitectus</i>	1344037	25526553		
	<i>ipsa</i>	2515736	27436711		
	<i>koira</i>	4829709	39140586		
<i>ROH/relatedness</i>	<i>lapistola</i>	2096897	15027126		
	<i>calcitectus</i>	28543726	59163910	840488	6321327
	<i>ipsa</i>	19696103	59423492	1102748	2330848
	<i>koira</i>	21253699	64929229	3096006	1995327
	<i>lapistola</i>	19006205	61841436	1754595	2116934
<i>PCA</i>	<i>nana group</i>	42350791	161819237		
	<i>kimberleyi</i>	7782572	69122761		
	<i>paranana</i>	8431248	27714038		
	<i>girloorloo</i>	8853097	59351243		
	<i>nana</i>	16278172	81813200		
<i>ROH/relatedness</i>	<i>kimberleyi</i>	24300280	72285541	6799560	2714743
	<i>paranana</i>	20549973	67352570	3663366	3614721
	<i>girloorloo</i>	35729728	68124115	7136591	7563665
	<i>nana</i>	36743496	78470964	13640400	4446596

Table S4. Average pairwise relatedness of samples within each species. Negative values indicate that individuals within each group are unrelated, as they share fewer alleles than expected by chance.

species	mean relatedness	min. relatedness	max. relatedness	range
<i>nana</i>	-0.999	-2.141	-0.051	widespread
<i>paranana</i>	-1.318	-3.694	0.019	short-range
<i>kimberleyi</i>	-0.180	-0.323	-0.049	widespread
<i>girloorloo</i>	-0.118	-0.316	0.008	short-range
<i>koira</i>	-0.164	-0.370	-0.020	widespread
<i>ipsa</i>	-0.136	-0.301	0.034	short-range
<i>calcitectus</i>	-0.157	-0.429	0.251	short-range
<i>lapistola</i>	-0.424	-1.141	-0.025	short-range

Table S5. Linear model of log-transformed evaporative water loss. Related to Figure 5.

Evaporative water loss (EWL, mg/min) is modelled as a function of body surface area (SA; cm²) and species (sequential sums of squares; SA first). A model including the SA × Species interaction was not significant ($F_{7,143} = 1.71$, $P = 0.110$), indicating a common log(EWL) ~ SA slope across species. df = degrees of freedom, SS = sum of squares, MS = mean square.

	Estimate	df	SS	MS	F-statistic	p-value
SA	0.0262	1	35.72	35.72	88.64	< 0.001
Species		7	7.94	1.13	2.82	0.009
Residual		150	60.45	0.40		

Table S6. Pairwise contrasts of surface area-adjusted evaporative water loss (EWL) among species. Related to Figure 5. Difference and standard error (SE) are on the log scale; ratio is the back-transformed difference (first species / second species); *p*-values are Tukey-adjusted.

Contrast	Difference (log)	SE	Ratio	<i>t</i> -statistic	<i>p</i> -value
<i>koira</i> – <i>lapistola</i>	0.417	0.244	1.52	1.71	0.683
<i>koira</i> – <i>ipsa</i>	0.127	0.234	1.14	0.54	0.999
<i>koira</i> – <i>calcitectus</i>	0.192	0.157	1.21	1.22	0.925
<i>koira</i> – <i>paranana</i>	−0.148	0.241	0.86	−0.62	0.999
<i>koira</i> – <i>nana</i>	0.458	0.225	1.58	2.04	0.462
<i>koira</i> – <i>girloorloo</i>	0.670	0.217	1.96	3.09	0.048
<i>koira</i> – <i>kimberleyi</i>	0.391	0.171	1.48	2.29	0.305
<i>lapistola</i> – <i>ipsa</i>	−0.290	0.309	0.75	−0.94	0.982
<i>lapistola</i> – <i>calcitectus</i>	−0.225	0.257	0.80	−0.88	0.988
<i>lapistola</i> – <i>paranana</i>	−0.565	0.320	0.57	−1.76	0.645
<i>lapistola</i> – <i>nana</i>	0.041	0.310	1.04	0.13	> 0.999
<i>lapistola</i> – <i>girloorloo</i>	0.254	0.304	1.29	0.84	0.991
<i>lapistola</i> – <i>kimberleyi</i>	−0.026	0.267	0.97	−0.10	> 0.999
<i>ipsa</i> – <i>calcitectus</i>	0.065	0.248	1.07	0.26	> 0.999
<i>ipsa</i> – <i>paranana</i>	−0.276	0.318	0.76	−0.87	0.989
<i>ipsa</i> – <i>nana</i>	0.331	0.308	1.39	1.07	0.961
<i>ipsa</i> – <i>girloorloo</i>	0.543	0.302	1.72	1.80	0.622
<i>ipsa</i> – <i>kimberleyi</i>	0.264	0.259	1.30	1.02	0.971
<i>calcitectus</i> – <i>paranana</i>	−0.340	0.252	0.71	−1.35	0.877
<i>calcitectus</i> – <i>nana</i>	0.266	0.235	1.31	1.13	0.948
<i>calcitectus</i> – <i>girloorloo</i>	0.479	0.228	1.61	2.10	0.417
<i>calcitectus</i> – <i>kimberleyi</i>	0.199	0.187	1.22	1.06	0.963
<i>paranana</i> – <i>nana</i>	0.607	0.242	1.83	2.50	0.203
<i>paranana</i> – <i>girloorloo</i>	0.819	0.238	2.27	3.45	0.017
<i>paranana</i> – <i>kimberleyi</i>	0.539	0.251	1.72	2.15	0.389
<i>nana</i> – <i>girloorloo</i>	0.212	0.201	1.24	1.06	0.965
<i>nana</i> – <i>kimberleyi</i>	−0.067	0.232	0.94	−0.29	> 0.999
<i>girloorloo</i> – <i>kimberleyi</i>	−0.280	0.224	0.76	−1.25	0.917

Table S7. Phylogenetic ANOVA (phylANOVA) showing that SA-adjusted EWL does not differ between widespread species and short-range endemics. Related to Figure 5. df = degrees of freedom, SS = sum of squares, MS = mean square, P_{phy} = the proportion of 10,000 simulations on the species tree yielding $F \geq$ the observed value.

	df	SS	MS	<i>F</i>-statistic	P_{phy}
SRE vs. widespread	1	0.0019	0.0019	0.022	0.894
Residual	6	0.504	0.084		