MSAP in Tiger Snakes: Island populations are epigenetically more divergent.

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Abstract:

Research on changes in phenotypic plasticity within wild animal populations is centuries old, however far fewer studies have investigated the role that epigenetics play in the development or persistence of natural variation in response to environmental change. Tiger snakes (*Notechis scutatus*) are an ideal study organism to investigate the link between epigenetics and phenotypic responses to environmental change, as they live on a range of offshore islands with different environments and prey types while exhibiting gigantism and dwarfism in body and head size. In this study, we have generated methylation sensitive amplified polymorphism (MSAP) data and found that, in general, Tiger Snakes are more epigenetically differentiated than genetically differentiated. Each island group has a distinctive epigenetic signal, suggesting the Tiger Snakes on each island group have adapted to their specific environment. This is also supported by the strong positive relationship between epigenetic differentiation and isolation age, as well as between epigenetic/genetic signal and both temperature and precipitation. The Tiger Snakes from Kangaroo Island, which has a complex landscape/environment like the mainland rather than the simple landscape/environment of each of the smaller islands, are both genetically and

epigenetically more like the mainland. As the MSAP loci are randomly distributed across the genome, we believe a closer examination of the epigenetic modifications near genes involved in growth, development, and lipid metabolism will allow us to investigate the epigenetic basis for the natural variation in head size and body size on the islands.

Introduction:

Evolutionarily adaptive responses in animals take place through a complex set of mechanisms that includes physical traits, behaviour, and environment but also the genes encoding for these characteristics and their epigenetic regulation. It has long been hypothesised that important fitness traits across a species, such as variation in body size and the tolerance of aridity and heat, may not be hard-wired in the DNA, but rather influenced by epigenetic modification to the DNA backbone that allows immediate responses to environmental cues (Feinberg & Irizarry 2011). Thus the phenotype of an organism appears to be influenced by epigenetic markers to some degree, with aberrations in these mechanisms creating variation that can lead both to adaptive and maladaptive traits (Consuegra and Rodriguez Lopez, 2016). DNA methylation is the best, and most easily, characterized epigenetic modification, and one that has been demonstrated to remain stable through the germline (Jablonka & Raz 2009).

Little is known about epigenetic variation in wild populations, how it is generated and how it relates to phenotypic plasticity. Island populations provide a source of natural variation, which can be used to assess naturally occurring epigenetic variation. An ideal species for examining epigenetic variation in animals in the Australian context is the Tiger Snake, as it occurs on multiple offshore islands that

have been isolated for varying amounts of time. The isolation of Tiger Snakes on Australia's offshore islands stem from both sea-level rise after the last glacial maximum (6-14,000 years ago [ya]) and the transport and release of captive populations by humans ~100 ya. In the recently isolated populations, neonate Tiger Snakes can adjust their head size to suit prey size (Aubret & Shine 2009), suggesting an epigenetic regulation of growth genes. Whereas on island populations with large prey items separated for thousands of years, the Tiger Snakes are born, and/or develop into, giant-sized snakes (Shine 1987), suggesting the traits governing body/head gigantism have been genetically assimilated. This well-characterised phenotypic plasticity makes the Tiger Snake an ideal wild species to investigate the link between epigenetic gene regulation and phenotypic plasticity.

In this study, we use methylation sensitive amplified polymorphism (MSAP) to characterise the differences in methylation between Tiger Snake populations and correlate with various population characteristics, such as geographic distance between populations, isolation age, and bioclimatic variables.

Methods:

DNA extractions

A total of 70 samples were obtained from the Australian Biological Tissue Collection (ABTC) at the South Australian Museum (SAM) or from the Australian National Wildlife Collection (ANWC) at the Commonwealth Scientific and Industrial Research Organisation (CSIRO). Sample geographic origin was determined from the specimen voucher information. In brief, a total of 38 samples were originally collected from island populations, including Kangaroo Island, Chappell Island, Nuyts Archipelago (Goat, Franklin and St Peter Islands) and Sir Joseph Banks Islands

(Roxby Island, Reevesby Island and Hareby Islands) while 32 samples where from continental populations (for detailed information see Supplementary Table 1). DNA was extracted from the tissue samples using either a 'salting-out' method (Nicholls *et al.* 2000) or a DNeasy Blood and Tissue kit (Qiagen).

Amplified Fragment Length Polymorphism (AFLP) and Methylation Sensitive Amplification Polymorphism (MSAP) Profiling

AFLP and MSAP methods were adapted from (Rodriguez Lopez *et al.* 2012). Briefly, 5.5 µl of the normalized gDNA were used for parallel DNA restriction and ligation performed using a combination of restriction enzyme *Eco*RI and *Mse*I for AFLPs and *EcoR*I and one of two isoschizomers *Msp*I or *Hpa*II for MSAPs. DNA was added to 5.5 µl the Restriction/Ligation master mix containing: 1.1 µl T4 Ligase buffer (x10), 1.1 µl Nacl (0.5 M), 0.55 µl BSA (1 mg/ml), 1U *Mse*I (New England Biolabs) or *Hpa*II (New England Biolabs) or *Msp*I (New England Biolabs), 5U *EcoR*I (New England Biolabs), 20U T4 DNA Ligase (New England Biolabs), 1 µl *EcoR*I adapter (5 µM), 1 µl *Hpa*II/*Msp*I adapter (50 µM) or 1 µl *Mse*I adapter (50 µM) (See Table 1 for full sequences), and water to a final reaction volume of 11 µl. Restriction/Ligation was performed using a T100™ Thermo cycler (Bio-Rad Laboratories, Hercules, CA) with the following protocol: 2 h at 37 °C followed by 10 min at 65 °C.

For the pre-amplification step, 1 µl of each Restriction/Ligation reaction product was added to 11.5 µl of pre-amplification master mix containing: 6.25 µl 2x Biomix (Bioline), 0.25 µl *Hpa*II/*Msp*I pre-amplification primer (10 µM) or 0.25 µl *Mse*I pre-amplification primer (10 µM), 0.5 µl *EcoR*I pre-amplification primer (10 µM) (Table 1), 0.1 µl BSA (1 mg/ml) and 4.85 µl Water. PCR amplification was

performed using a T100^{TM} Thermo cycler (Bio-Rad Laboratories, Hercules, CA) with the following protocol: 2 min at 72 °C, followed by 30 cycles of 30 sec at 94 °C, 30 sec at 56 °C, 2 min at 72 °C with a final step of 10 min at 72 °C.

A second round of selective amplification was carried out by adding 1 µl of the Pre-amplification PCR product to 11.5 µl of the Selective amplification Master mix containing: 6.25 µl 2x Biomix (Bioline), 0.25 µl 5'-FAM labelled *Hpa*II/*Msp*I Selective primer (10 µM) or 0.25 µl 5'-FAM labelled *Mse*I Selective primer (10 µM), 0.5 µl *EcoR*I Selective primer (10 µM) (Table 1), 0.1 µl BSA (1 mg/ml) and 4.85 µl nanopure water. PCR amplification was performed using a T100^{TM} Thermo cycler (Bio-Rad Laboratories, Hercules, CA) with the following protocol: 2 min at 94 °C, followed by 13 cycles of 30 sec at 94 °C, 30 sec at 65 °C (reduce by 0.7 ˚C per cycle), 2 min at 72 °C, followed by 24 cycles of 30 sec at 94 °C, 30 sec at 56 °C, 2 min at 72 °C with a final step of 10 min at 72 °C. Selective amplification products were sent for capillary electrophoresis separation to the Australian Genome Research Facility Ltd (AGRF), Adelaide, South Australia.

Statistical analysis

MSAP profiles were visualized using GeneMapper Software v4 (Applied Biosystems, Foster City, CA). Binary matrices containing presence (1) absence (0) allelic information were generated from the capillary separation results obtained from samples restricted using enzyme combinations *Eco*RI/*Mse*I (AFLPs) and *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II (MSAPs). In this case, fragment selection was limited to allelic sizes between 85 and 550 bp to reduce the potential impact of size homoplasy (Caballero *et al.* 2008). In all cases, different levels of hierarchy were generated to group the samples. Samples were first grouped into continental or island populations.

125 Then, samples from island populations were divided according to the island they were
126 collected from. Finally, samples from island populations were separated by island age.
127 The numbers of the various fragments attributed to non-methylated (+/+),
128 CHG methylated (+/-), CG methylated (-/+), and uninformative (-/-) were calculated
129 for each population based on the MSAP profiles. Shannon Diversity indices were
130 used to estimate the within-population (at the island group level) epigenetic diversity
131 (H_{pop}) of the CG and CHG methylation patterns and the Kruskal-Wallis H test was
132 used to estimate the significance of the differences in Shannon diversity index among
133 these populations.
134 For the analysis of the AFLP and MSAP data, GenAlex v6.5 software (Peakall
135 & Smouse 2012) was used to infer pairwise genetic (PhiPT calculated from AFLP
136 profiles) and epigenetic (ePhiPT calculated from MSAP profiles) distances between
137 the different hierarchical groups described above. Analysis of Molecular Variance
138 (AMOVA) was then performed using the same software to test the significance of the
139 estimated distances between groups using 9999 random permutations. Principal
140 Coordinates Analysis (PCoA) was used to visualize the patterns of epigenetic
141 variation associated to geographical origin and island age. The PCoA were performed
142 in the R package 'stats' using the prcomp command.
143 Mantel test analyses were then used to estimate the correlation between: 1) the
144 calculated pairwise molecular distances (i.e. PhiPT and ePhiPT) and the pairwise
145 geographic distance in km (GGD) among populations and 2) the calculated pairwise
146 molecular distances (i.e. PhiPT and ePhiPT) and the pairwise age differences among
147 populations as described by Rois *et al.* (2013). Mantel test significance was assigned
148 as the probability of the correlation between compared matrices using random data
149 being higher than the correlation between matrices of collected data (i.e. P(rxy-rand

>= rxy-data)) estimated over 9,999 random permutations tests, as implemented in Genalex v6.5.

Climate

The 19 bioclim layers were downloaded from the worldclim website (worldclim.org/bioclim), converted from tiff to ascii files and trimmed to focus on Australia using the R computing package Raster. The values of the 19 bioclim layers for each of the South Australia sample locations used in this study were extracted from the climate data and input into a principal component analysis (PCA) to examine how each population varies in overall climate. Mantel test analyses (R package ade4) were also used to estimate the correlation between differences in epigenetic/genetic principal components (i.e. PC1 of the MSAP PCoA) and each of the 19 bioclimatic variables for the sampling locations within South Australia (where most of the samples were collected from) and converted to a distance matrix. The relationships between these raw datasets were also plotted with regression lines and Pearson correlation coefficients were calculated to estimate the strength of these relationships using the R package 'stats'.

Results:

67 Tiger Snake samples were successfully genotyped, with 38 from island populations and 29 from mainland Australia. As there were not enough successfully genotyped samples from some locations to generate population level data, the majority of analyses were performed on samples from South Australia: the Nuyts Archipelago (n=12), Sir Joseph Banks Island group (n=11), Kangaroo Island (n=7) and the South Australian mainland (n=17; see Figures 1 & 2 for sampling locations).

The numbers of the various fragments attributed to non-methylated (+/+), CHG methylated (+/-), CG methylated (-/+), and uninformative (-/-) were calculated for the mainland populations and island populations separately based on the MSAP profiles. Within South Australia (SA), the total 5'-CCGG-methylation level (both CG and CHG together) ranged from 22% (of fragments) in mainland SA Tiger Snakes to 30% on Roxby Island (Table 2 & Figure 3). The amongst SA population difference in overall genome-wide methylation levels (both CG and CHG methylation together) was not significant (ANOVA p = 0.6270), nor was the difference in CHG methylation between SA populations (ANOVA p = 0.1637); however the difference in CG methylation between each of the SA populations was significant at the 5% level (ANOVA p = 0.0341; range from 10% of fragments on Goat Island to 17% on Roxby Island; Table 2). Shannon diversity indices were calculated for each methylation type per SA population group (Table 3) and Kruskal-Wallis rank sum tests were used to test for differences between Tiger Snakes on the SA mainland and each of the island groups, which were significantly different at the 10% level for CG methylation (chi-squared = 2.9503, p = 0.08586), and for CHG methyation (chi-squared = 3.3103, p = 0.06884). The coefficient of epigenetic differentiation was also higher in the SA mainland populations compared to the SA island populations (Table 4.)

Genetics

The genetic PCoA shows that some Kangaroo Island Tiger Snake samples are genetically closer to mainland samples, while others are genetically closer to island populations (Figures 4 & 5). Within SA the populations that are genetically closest to each other are the Sir Joseph Banks Island group and Nuyts Archipelago populations (Nei genetic distance = 0.031; PhiPT = 0.041; Table 5) and they appear intertwined on the PCoA (Figure 5). Using Nei's genetic distance, the Kangaroo Island and

mainland Tiger Snakes are also genetically quite close (Nei genetic distance = 0.034; PhiPT = 0.024), with the most genetically distant populations the mainland-Nuyts Archipelago populations (Nei genetic distance = 0.072; PhiPT = 0.166).

Epigenetics

The populations that are epigenetically closest to each other are the mainland/KI populations (using both Nei's and PhiPT epigenetic distance for the HpaII dataset) and the Sir Joseph Banks/Nuyts Archipelago populations (Nei's and PhiPT epigenetic distance for the MspI dataset; Tables 6 & 7). The populations that are epigenetically most distant are the Sir Joseph Banks islands vs. mainland (*Msp*I PhiPT epigenetic distance) and the Sir Joseph Banks islands vs. all the other populations (HpaII PhiPT epigenetic distance). The Sir Joseph Banks Island group are comprised of Roxby Island, Reevesby Island and Hareby Islands, which each also had the three highest overall percentages of fragments that were methylated (Table 3). The epigenetic PCoA of all Tiger Snake samples, again shows how some Kangaroo Island samples resemble those on the mainland, but others resemble those on the smaller nearby islands (Figure 6).

When only South Australian (SA) Tiger Snakes were examined, the SA mainland samples overlapped to a large degree with those on Kangaroo Island and were quite distinct from those on the Nuyts Archipelago and Sir Joseph Banks Island group in PC1 (Figure 7). Values in PC2, on the other hand, separated islands from the Nuyts Archipelago from those in the Sir Joseph Banks Island group, with the mainland and Kangaroo Island populations in between (Figure 8).

There is also strong correlation ($R^2 = 0.4797$ in Figure 9) between genetic distance and geographic distance (traditionally termed isolation-by-distance), however the correlation between epigenetic distance and geographic distance is stronger ($R^2 =$

224 0.5468 in Figure 10). An even stronger correlation also occurs between epigenetic
225 distance and isolation age (R^2 = 0.5673 in Figure 11 and R^2 = 0.6922 in Figure 12).
226
227 **Environmental data**
228 There are some clear environmental differences between some of the Tiger
229 Snake populations (Figures 13 & 14). The two main bioclimatic variables driving the
230 PCA relationships between the populations are BIO4 (temperature seasonality) and
231 BIO12 (annual precipitation). When we look at how these bioclimatic variables are
232 related to epigenetic distance, we find that similar to the strong correlation between
233 epigenetic distance and isolation age, there are also even stronger correlations
234 between epigenetic distance and bioclimatic variables for the South Australian (SA)
235 samples. The SA mainland samples generally experience higher annual mean
236 temperatures (~15.7—17.3 °C) compared to Tiger Snakes on the Nuyts Archipelago
237 and Sir Joseph Banks islands (~13.5—16.3 °C), which appears to be strongly related
238 (r = 0.652) to their genetic/epigenetic profiles (*Hpa*II PCoA1 values; Figure 15 & 16).
239 The SA mainland samples generally experience lower precipitation levels in the driest
240 month (< ~12mm) compared to Tiger Snakes on the Nuyts Archipelago and Sir
241 Joseph Banks islands (> ~12mm), which again appears to be strongly related (r = -
242 0.711) to their genetic/epigenetic profiles (*Hpa*II PCoA1 values; Figures 17 & 18).
243 Other bioclimatic variables related to mean temperature and precipitation levels are
244 also strongly associated with PC1 in the *Hpa*II PCoA, however Bio1 and Bio14 have
245 the strongest correlation (Table 8).
246
247 **Discussion:**

248 The MSAP technique only detects methylation at 5'-CCGG-3' sites and
249 cannot discriminate between methylation and fragment absence when both cytosines
250 are hypermethylated: therefore the level of genomic DNA methylation may be
251 underestimated. The *Hpa*II and *Msp*I datasets also have the added limitation of
252 possibly still retaining some genetic signal, which complicates the epigenetic patterns.
253 Bearing in mind these inherent limitations of the technique, our study has explored the
254 level and pattern of genome-wide 5'-CCGG-methylation in Tiger Snake island and
255 mainland populations.
256 When we compare the purely nuclear fragment length (AFLP) data, we see
257 similar patterns to that observed in mtDNA (Keogh et al. 2005): kangaroo island
258 Tiger Snakes fall with SA mainland samples and sister to some of the island samples
259 (Sir Joseph Banks Island group). The epigenetic (*Hpa*II) data adds an extra dimension
260 to the information about the Kangaroo Island Tiger Snakes, in that they look like
261 mainland populations both genetically (AFLP) and epigenetically (*Hpa*II). This could
262 be because of the relatively large size of the island and/or the complex
263 climate/habitat/prey types etc. The low levels of within-population epigenetic
264 diversity on KI suggests that even given the more complex island environment, the
265 Tiger Snakes on KI have all responded epigenetically in a similar way to each other
266 and to those on the mainland. A large complex environment may allow enough
267 heterogeneous habitats that behavioural adaptation (*i.e.* via migration to new
268 environments) is favoured over epigenetic *in-situ* adaptation that is the only option in
269 range and environmentally restricted habitats, such as those that occur on small
270 isolated islands.
271 There is more epigenetic differentiation within the mainland population as a
272 whole compared to that within the islands as a group again possibly because the

environment on the mainland is more heterogeneous than across the islands (Table 5). Another suggestion that the epigenetic signal is related to complex environmental differences is the fact that the Nuyts Archipelago and Sir Joseph Banks Island group are similar genetically to each other but significantly different in epigenetic signal even though they have been isolated for similar amounts of time and from presumably the same source population (Table 4, 6 & 7).

Even taking into account some retained genetic signal in PC1 of the *Hpa*II PCoA plots, these plots show interesting patterns: 1) the SA mainland populations are quite distinct from the Nuyts Archipelago and Sir Joseph Banks islands in PC space, which is not seen in the purely genetic data; 2) the intra-population variation (*i.e.* degree of clustering of each island population) is smaller in the epigenetic PCoA than in the genetic PCoA; and 3) the correlation between epigenetic and geographic distance is stronger than between genetic and geographic distance. If there is retained genetic signal within PC1 of the *Hpa*II PCoA (and of a similar nature to the genetic signal in the AFLP PCoA), then this retained genetic signal would in essence be adding noise to the epigenetic signal, suggesting the purely epigenetic signal should exhibit stronger separation between the mainland and island populations, even less intra-population variation in the island populations and stronger correlation between epigenetic distance and geographic distance than that observed. Unless the *Hpa*II cut sites present in this data occur purely by coincidence in adaptive genes, then these patterns suggest that the island populations have adapted through epigenetic mechanisms to their specific environments.

This idea of Tiger Snake adaptation to specific environments epigenetically is further reinforced by the very strong correlation between PC1 in the *Hpa*II PCoA with various temperature and precipitation bioclimatic variables. The size of each island,

298 as well as the habitat and climate, all possibly play a role in how adaptable the Tiger
299 Snakes need to be in terms of life history traits. There are distinct and quite different
300 ecological niches on the islands of the Nuyts Archipelago and the Sir Joseph Banks
301 Island group (colder and with more rain) compared to the mainland (warmer and with
302 less rain). The islands of the Sir Joseph Banks Island group and Nuyts Archipelago
303 are also generally small in size, with low profiles and little natural water (Robinson *et*
304 *al.* 1996), suggesting that uncharacteristically hot and dry seasons might have a larger
305 impact on the habitat and prey items of the Tiger Snakes on these islands.
306 Furthermore, on small islands, Tiger Snakes may need to retain flexibility in
307 body/head size development strategies in order to adapt to rapid and uncommon
308 weather events, as well as in changes in habitat and/or prey as they can't simply
309 migrate to follow their preferred niche and prey items. For example, if seasonal
310 storms wipe out mutton-bird nests, a lack of large high calorie prey items at a critical
311 time of year may result in only small reptiles being available for neonates as they
312 develop in their first year of age, with reduced gigantism one possible outcome
313 through epigenetic down-regulation of growth genes.
314 Interestingly, a strong correlation occurs between epigenetic distance (PhiPT
315 in *Hpa*II) and isolation age. This is counter-intuitive if epigenetic regulation of traits
316 develops over short time frames and then these traits get genetically assimilation over
317 longer time frames. Another possible explanation for this correlation between
318 epigenetic distance and isolation age is that the retained genetic signal has been
319 subject to neutral genetic drift, which might also appear as increasing epigenetic
320 differentiation relative to isolation time. The fact that Tiger Snake epigenetic patterns
321 are not that similar to the purely genetic patterns makes neutral drift an unlikely
322 explanation however. Neutral drift has been posited as one explanation for significant

323 correlation between genetic and epigenetic patterns in another MSAP study of wild
324 animal populations, however they suggest that methylation variation being dependent
325 on DNA sequence differences is a more likely explanation (Liu 2012). In the case of
326 the Tiger Snake, the environmental variability found on the SA islands may be too
327 extreme to select for a new optimal set of traits after isolation. This may necessitate
328 continued epigenetic regulation of phenotypic plasticity over thousands of years rather
329 than only an initial adaptive period after isolation followed by genetic assimilation as
330 per the model explaining epigenetic regulation of genes as a method of rapid
331 adaptation to novel environments.

332 **Conclusion:**

333 In conclusion, by randomly subsampling the genome and epigenome of the
334 Tiger Snake we found that Kangaroo Island Tiger Snakes resemble those on the
335 mainland both genetically and epigenetically to a large degree, possibly because both
336 KI and the mainland are environmentally complex. More importantly, we have also
337 shown that the SA island populations have adapted to their environment in terms of
338 both specific temperature and precipitation levels. Future work in this sphere needs to
339 use finer-scale methods to examine both the genetic and epigenetic signal across
340 populations of the Tiger Snake. Furthermore, a closer examination of epigenetic
341 regulation of specific genes involved in growth (*i.e.* for head and body size) and lipid
342 metabolism are needed to see epigenetic differences related to prey item variance on
343 the different islands and in comparison to the mainland Tiger Snakes.
344

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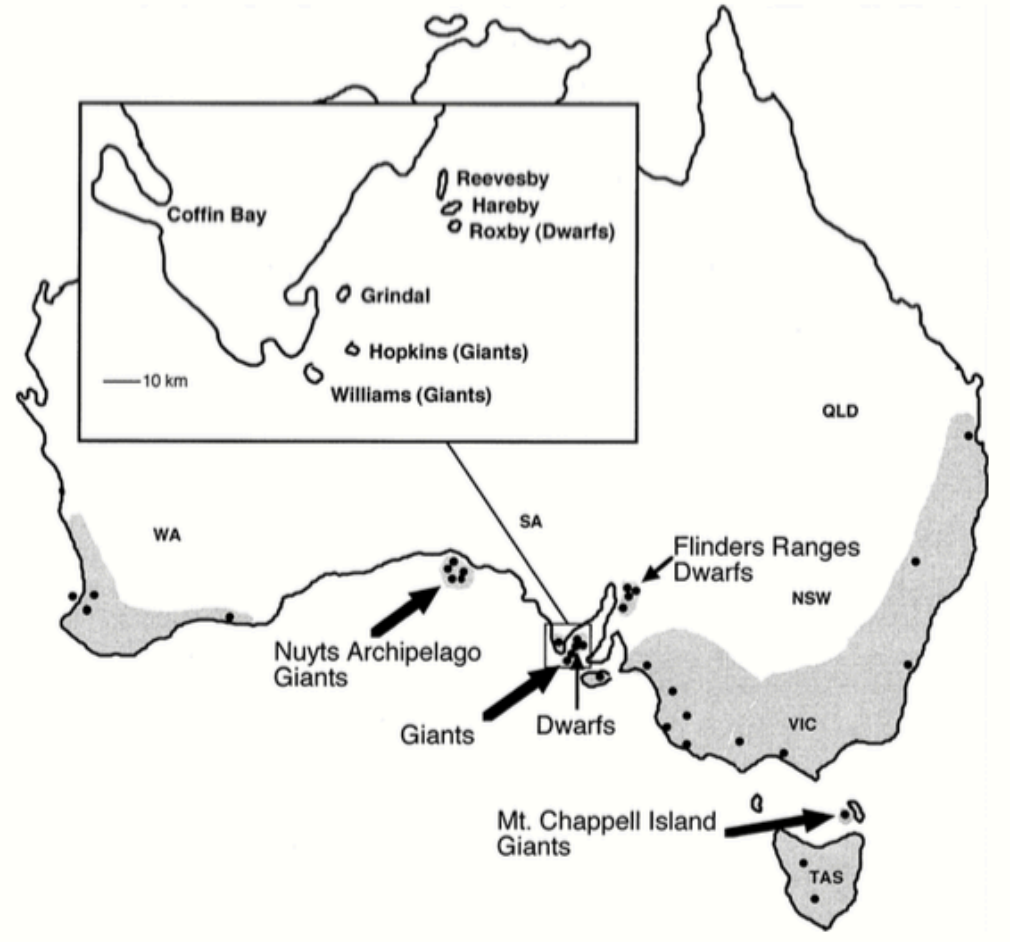
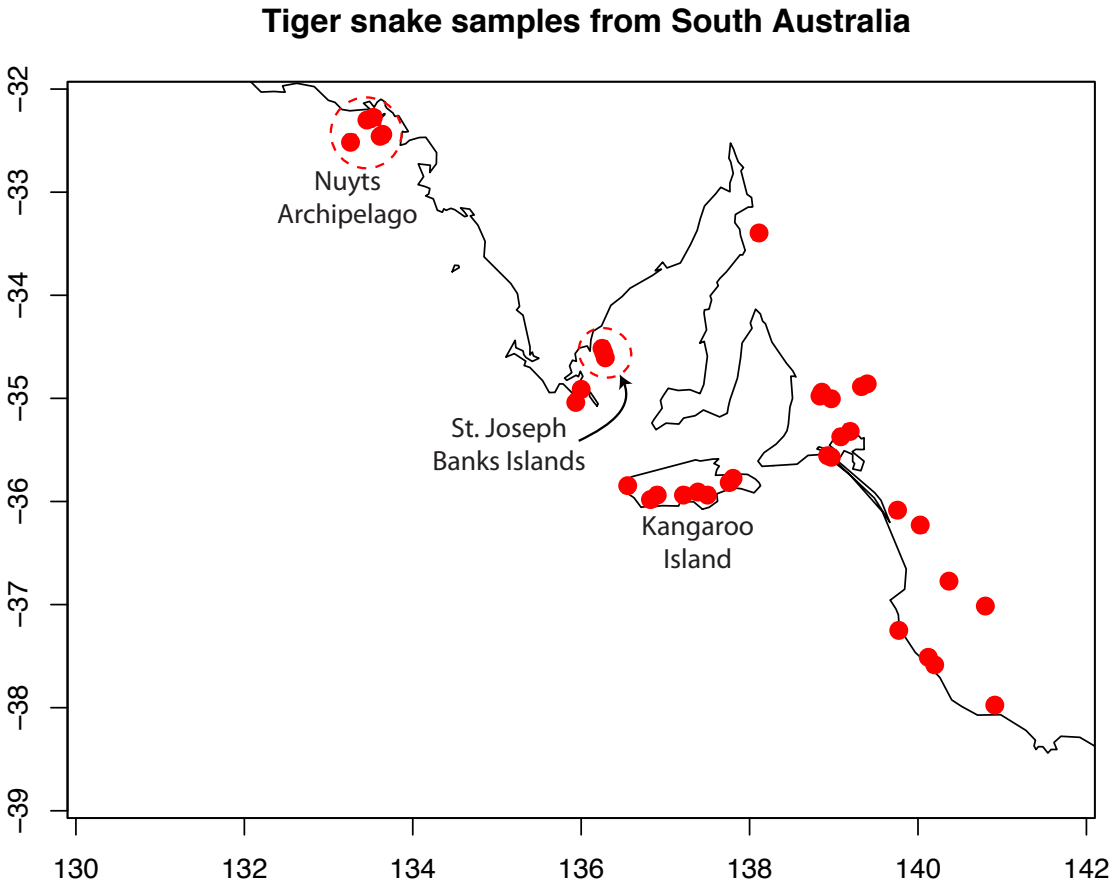
379 **Tables and Figures**



380

381 Figure 1. Map of Australia showing Tiger Snake populations sampled in this study

382 (redrawn from Keogh *et al.* 2005).



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384 Figure 2. Map of South Australia showing sampling locations and island groups.

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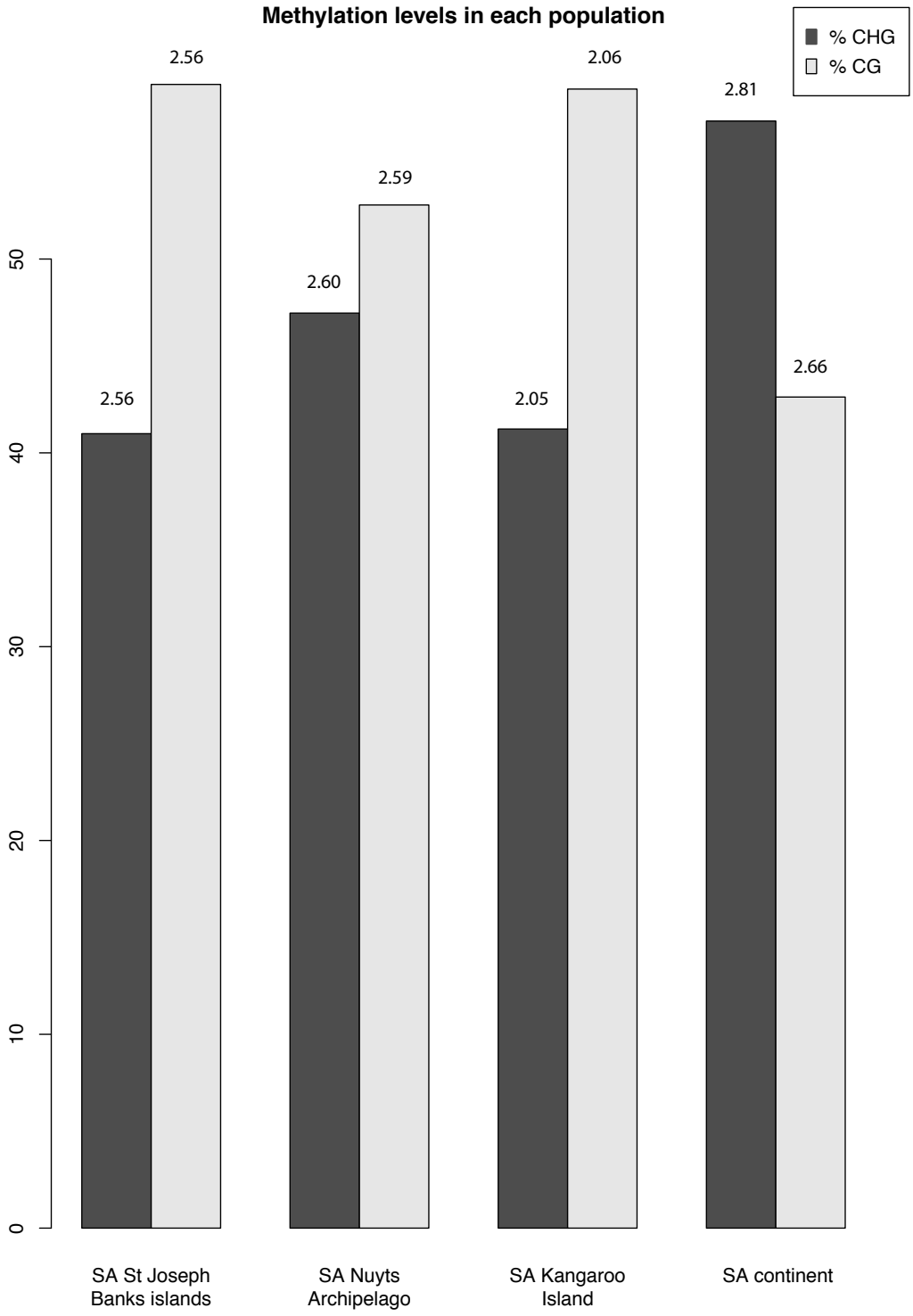

386

387 Figure 3. Histogram of CG and CHG methylation levels as a percentage of the total

388 methylated fragments in the South Australia mainland vs. island Tiger Snakes. Values

389 on top of the histogram bars are the Shannon Diversity Indices for within-population

390 epigenetic diversity (H_{pop}).

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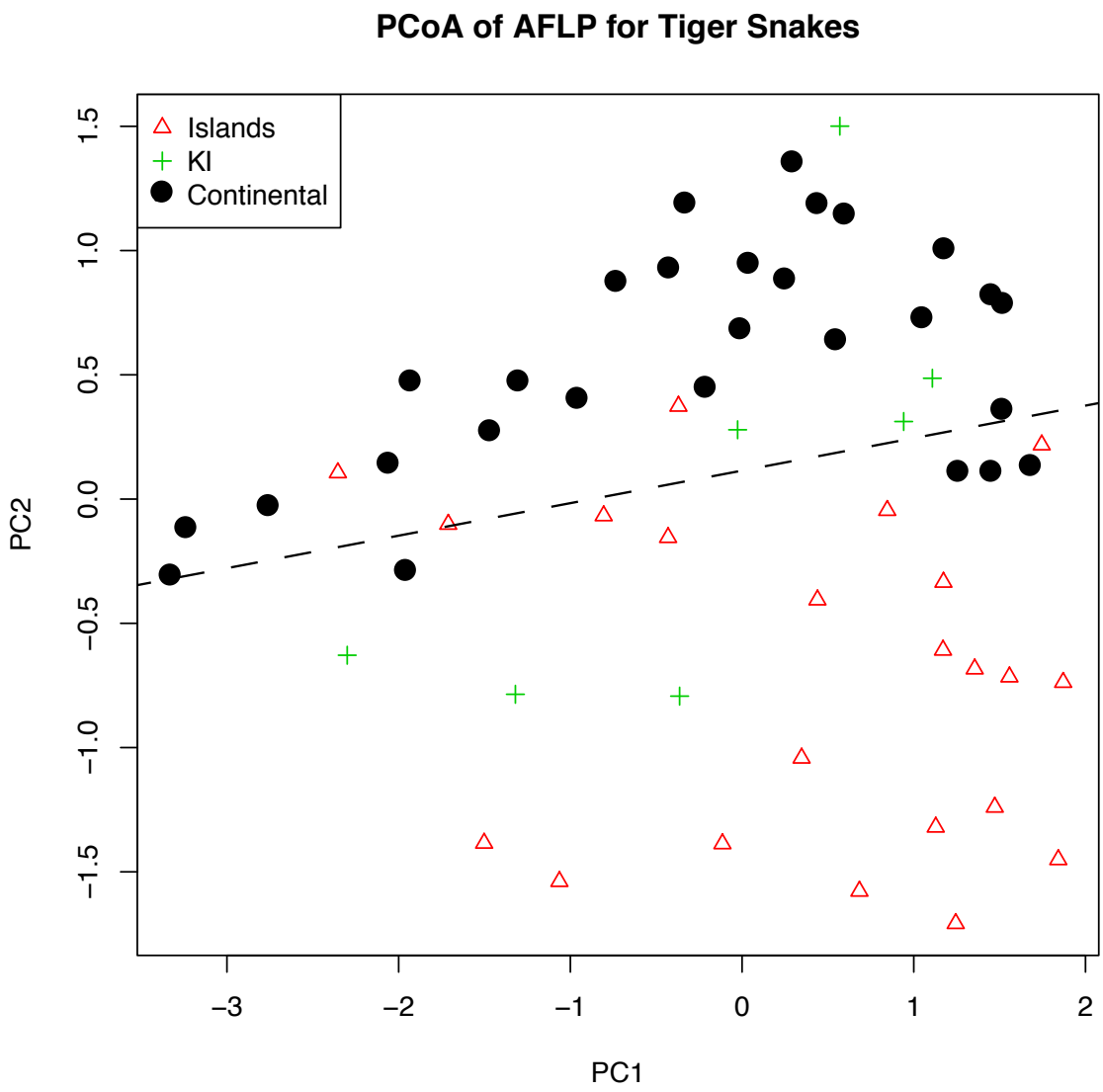

392

393 Figure 4. Principal co-ordinate analysis of genetic data (AFLP) for all Tiger Snake

394 samples, showing the major differentiation in PC2 of mainland (black circles) vs.

395 island (red triangles) populations. Some Kangaroo island samples (green crosses) fall

396 with the island populations but others fall with the mainland samples.

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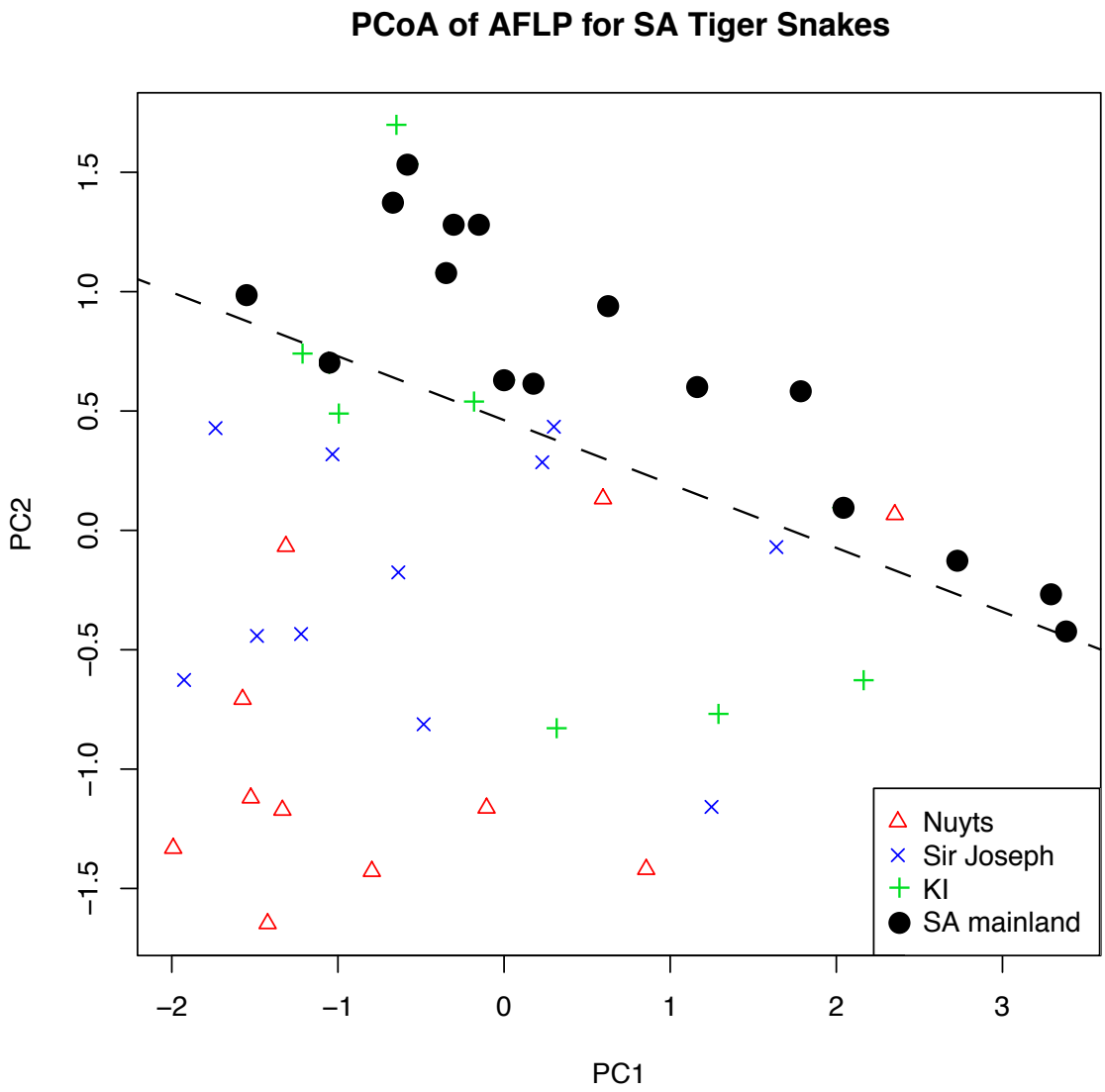


399

400 Figure 5. Principal co-ordinate analysis of genetic data (AFLP) for South Australian

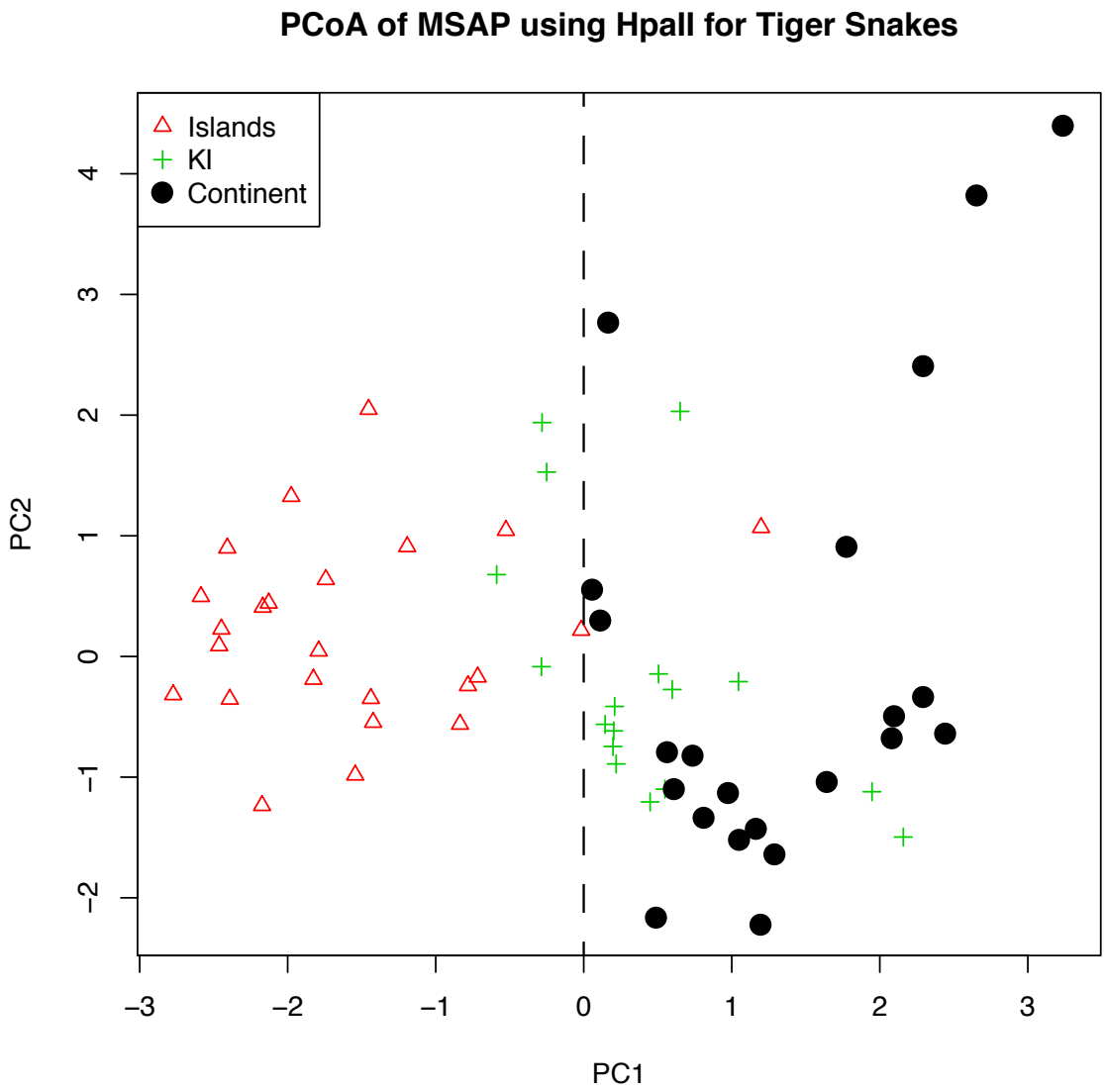
401 Tiger Snake samples only (n=59), showing the major differentiation in PC2 of

402 mainland (green plus sign) vs. Sir Joseph Banks island group (blue cross) vs. Nuyts

403 Archipelago (red triangle) populations. Again, some Kangaroo island samples (black

404 circle) fall with the other two island group populations but others fall with the

405 mainland samples.



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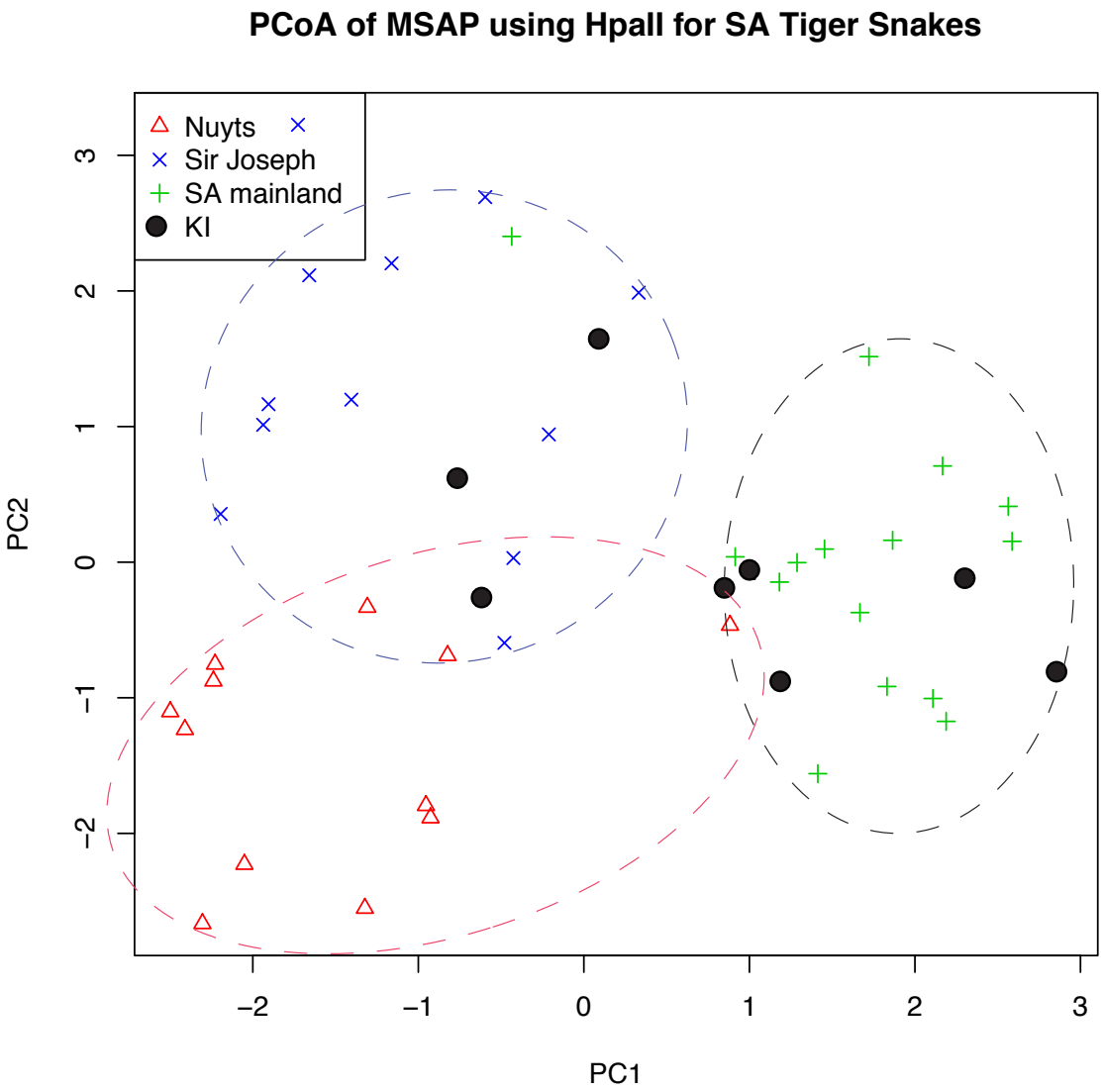
407 Figure 6. Principal co-ordinate analysis of the epigenetic markers (*Hpa*II) for all the

408 Tiger Snake samples (n=70), showing the first and second principal components.



409

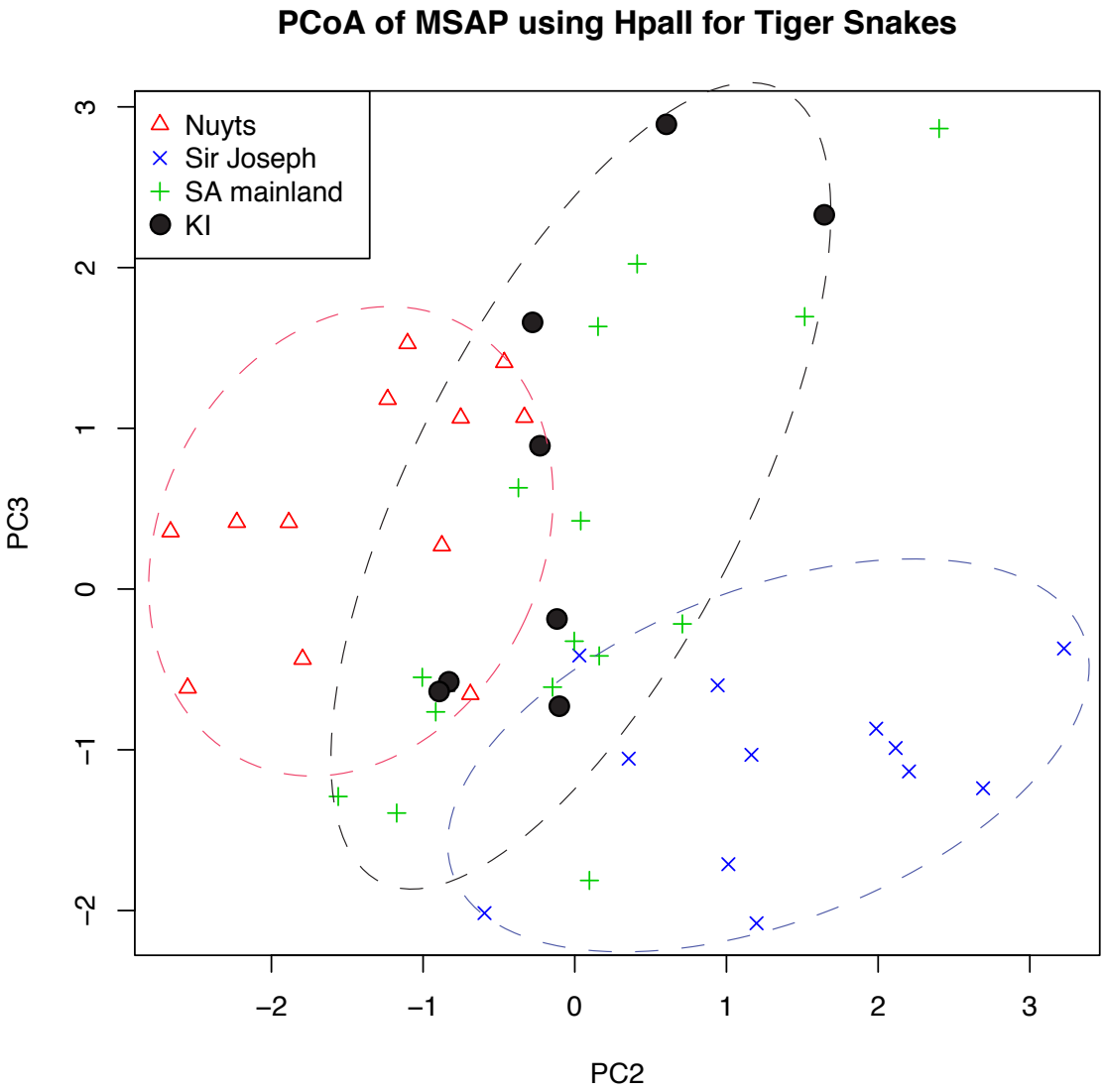
410 Figure 7. Principal co-ordinate analysis (PCoA) of the MSAP markers for the Tiger

411 Snake samples from South Australia (n=51), showing the first and second principal

412 co-ordinates. PC1 values from this PCoA were used in the mantel and correlation tests

413 to assess the relationships between the epigenetic/genetic signal and bioclimatic

414 variables.



415

416 Figure 8. Principal co-ordinate analysis of the MSAP markers for the Tiger Snake

417 samples from South Australia (n=51), showing the second and third principal co-

418 ordinates.

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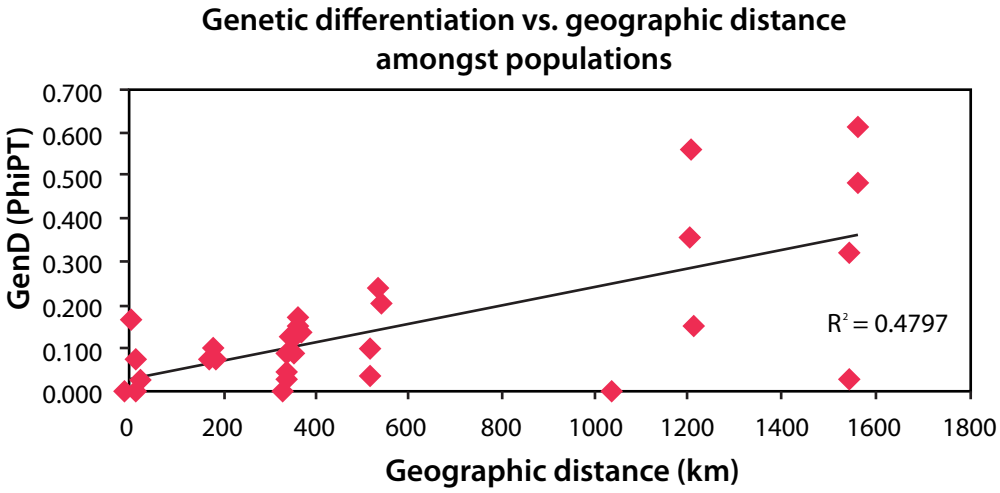
420

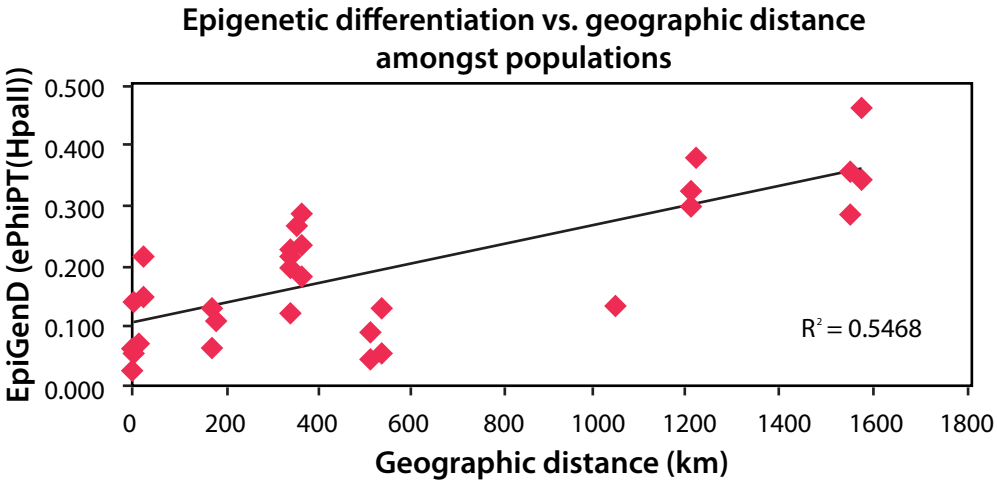


421

422 Figure 9. Mantel test of genetic differentiation against geographic distance amongst

423 all the populations (mantel p-value = 0.010).

424



425

426 Figure 10. Mantel test of epigenetic differentiation against geographic distance

427 amongst all the populations (mantel p-value = 0.010).

428

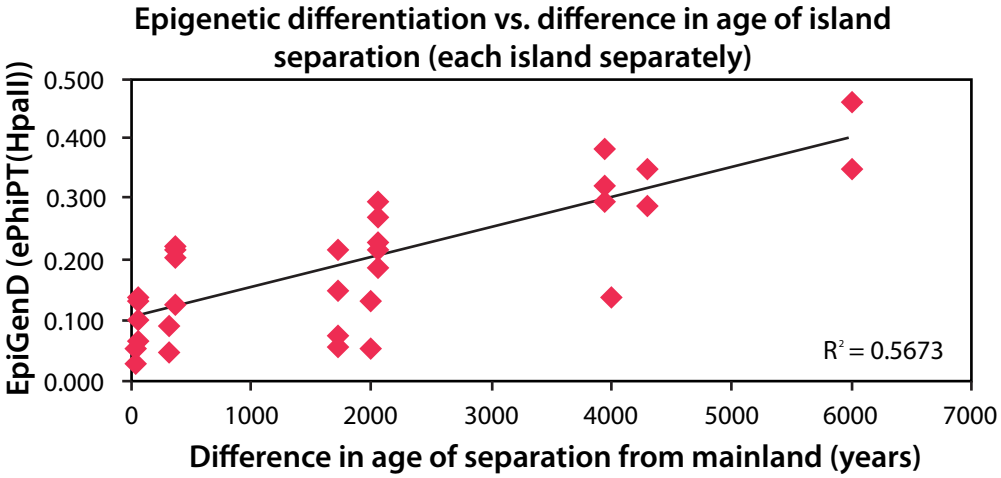


Figure 11. Mantel test of epigenetic differentiation against time since isolation between all the populations (mantel p-value < 0.001).

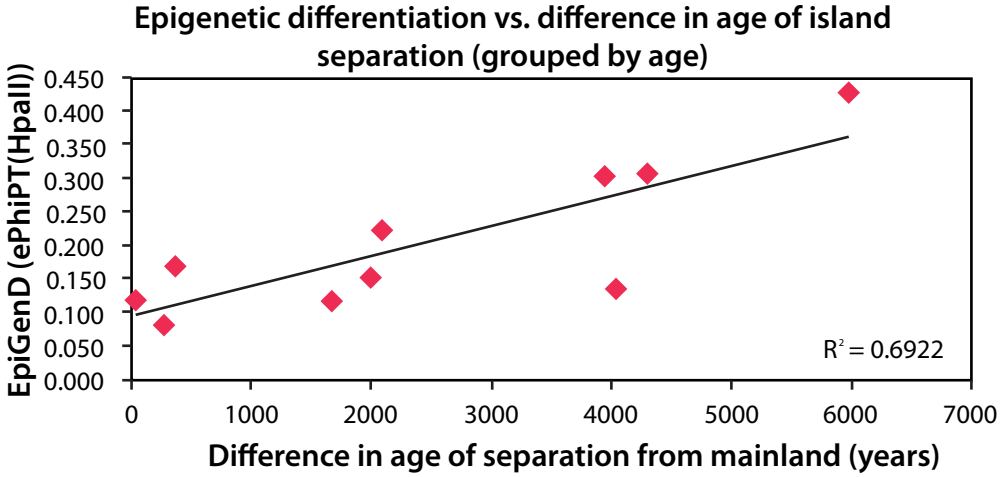


Figure 12. Mantel test of epigenetic differentiation against time since isolation between all the populations grouped by age (mantel p-value = 0.015).

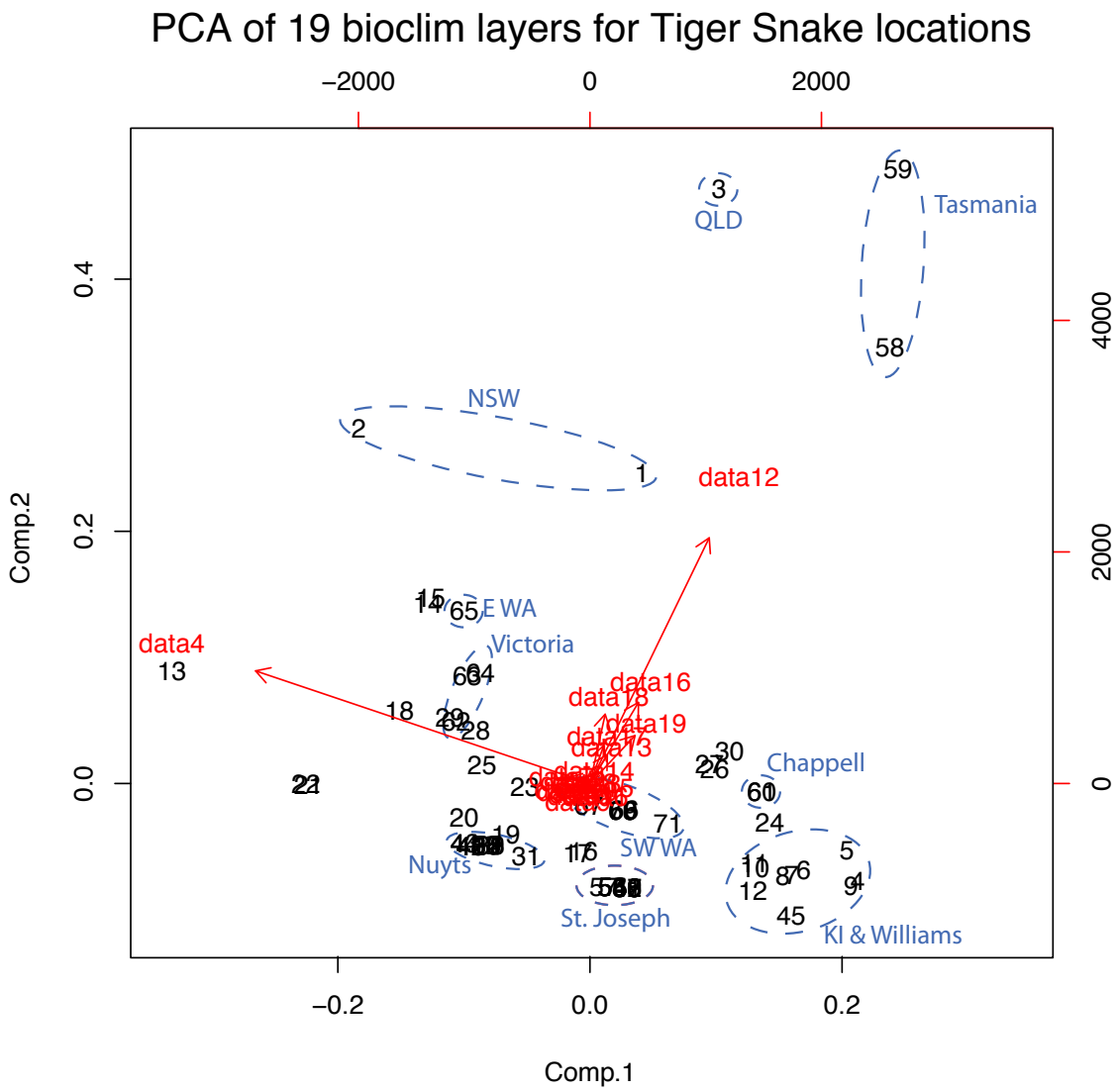


Figure 13. Principal component analysis (PCA) of 19 bioclimatic layer values for the all Tiger Snake sampling locations. The climate layer responsible for most of the variation in principal component 1 is BIO4, which is temperature seasonality, and the climate layer responsible for most of the variation in principal component 2 is BIO12, which is annual precipitation.

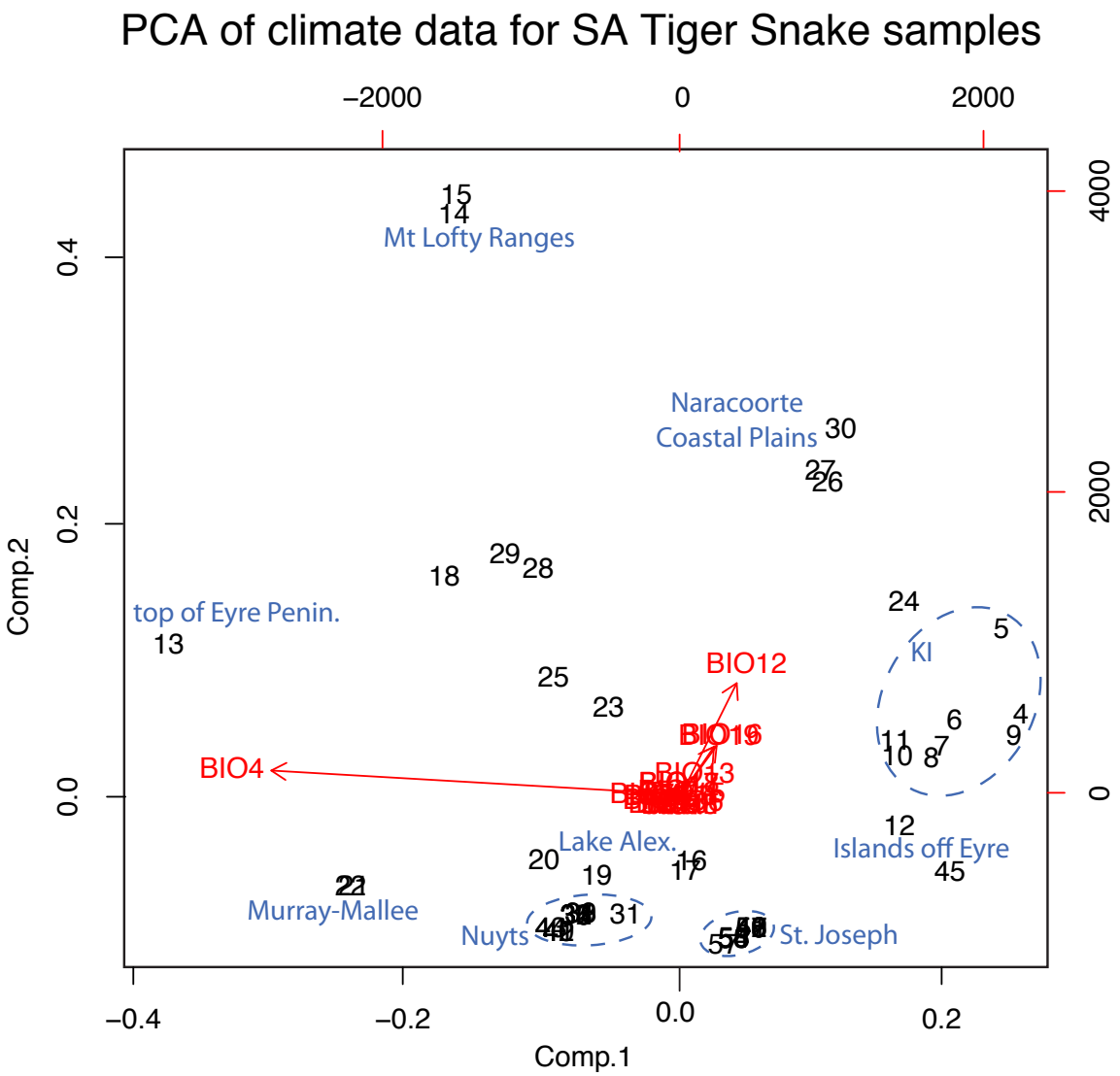


Figure 14. Principal component analysis (PCA) of 19 bioclimatic layer values for the South Australia Tiger Snake sampling locations. The climate layer responsible for most of the variation in principal component 1 is BIO4, which is temperature seasonality, and the climate layer responsible for most of the variation in principal component 2 is BIO12, which is annual precipitation.

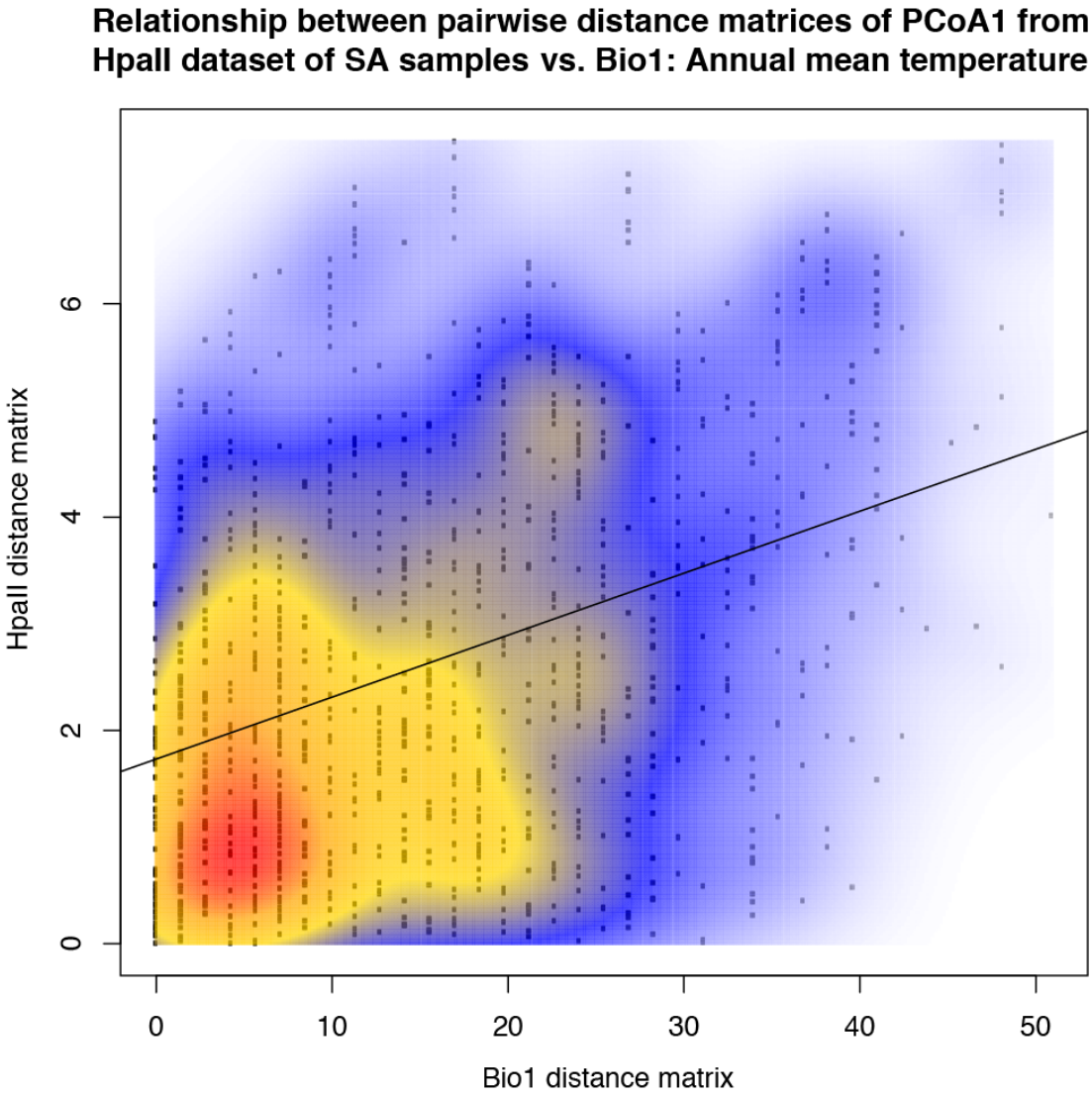


Figure 15. Mantel test showing correlation between pairwise distance matrix of *Hpa*II PCoA1 values and pairwise distance matrix of Bio1 variables (Annual mean temperature) for Tiger Snakes from South Australian locations. The correlation is significantly different from random, with a p-value of 0.001 (Bonferroni corrected p-value of 5% significance level is 0.0026).

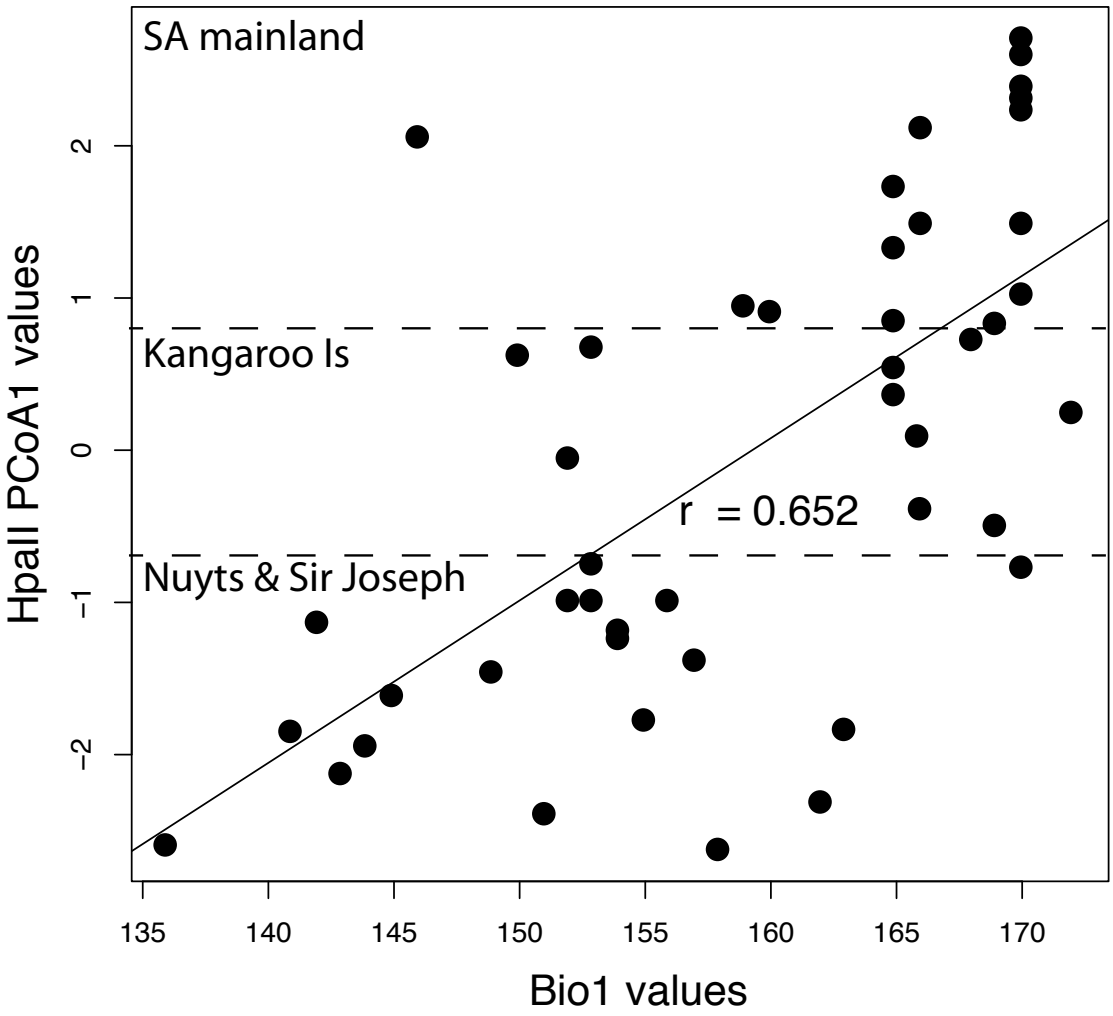


Figure 16. Scatterplot of raw *Hpa*II PCoA1 values vs. Bio1 (annual mean temperature) values for Tiger Snakes from South Australian (SA) locations. The horizontal dashed lines roughly separate out the SA mainland Tiger Snakes, from those on Kangaroo Island, and those on islands in the Nuyts Archipelago and Sir Joseph Banks Island group. The SA mainland samples generally experience higher annual mean temperatures (~15.7—17.3 °C) compared to Tiger Snakes on the Nuyts Archipelago and Sir Joseph Banks islands (~13.5—16.3 °C), which appears to be related to their genetic/epigenetic profiles (*Hpa*II PCoA1 values). The Pearson correlation coefficient (r = 0.652) shows a strong positive linear correlation.

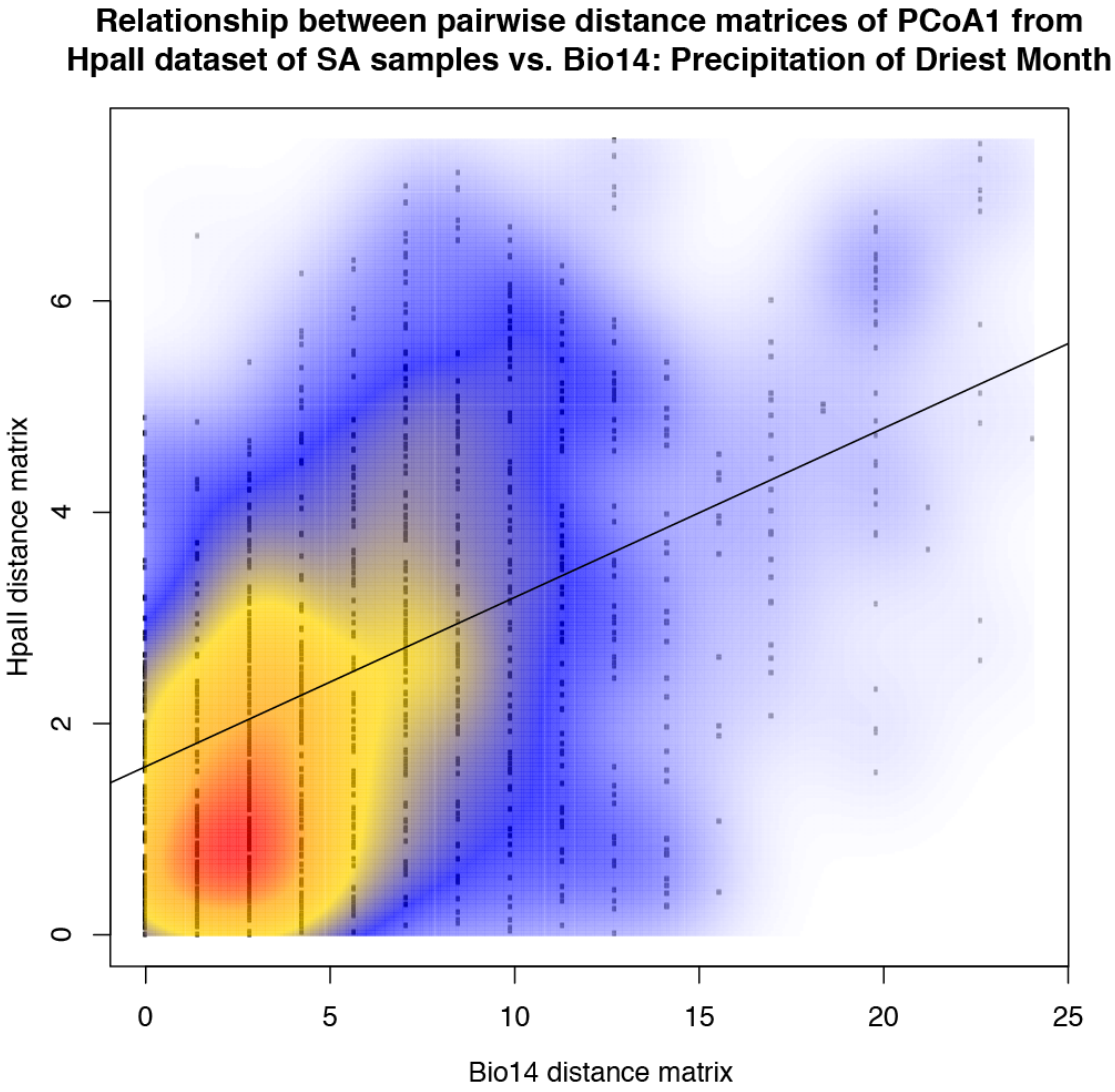


Figure 17. Mantel test showing correlation between pairwise distance matrix of *Hpa*II PCoA1 values and pairwise distance matrix of Bio14 variables (precipitation of the driest month) for Tiger Snakes from South Australian locations. The correlation is significantly different from random, with a p-value of 0.001 (Bonferroni corrected p-value of 5% significance level is 0.0026).

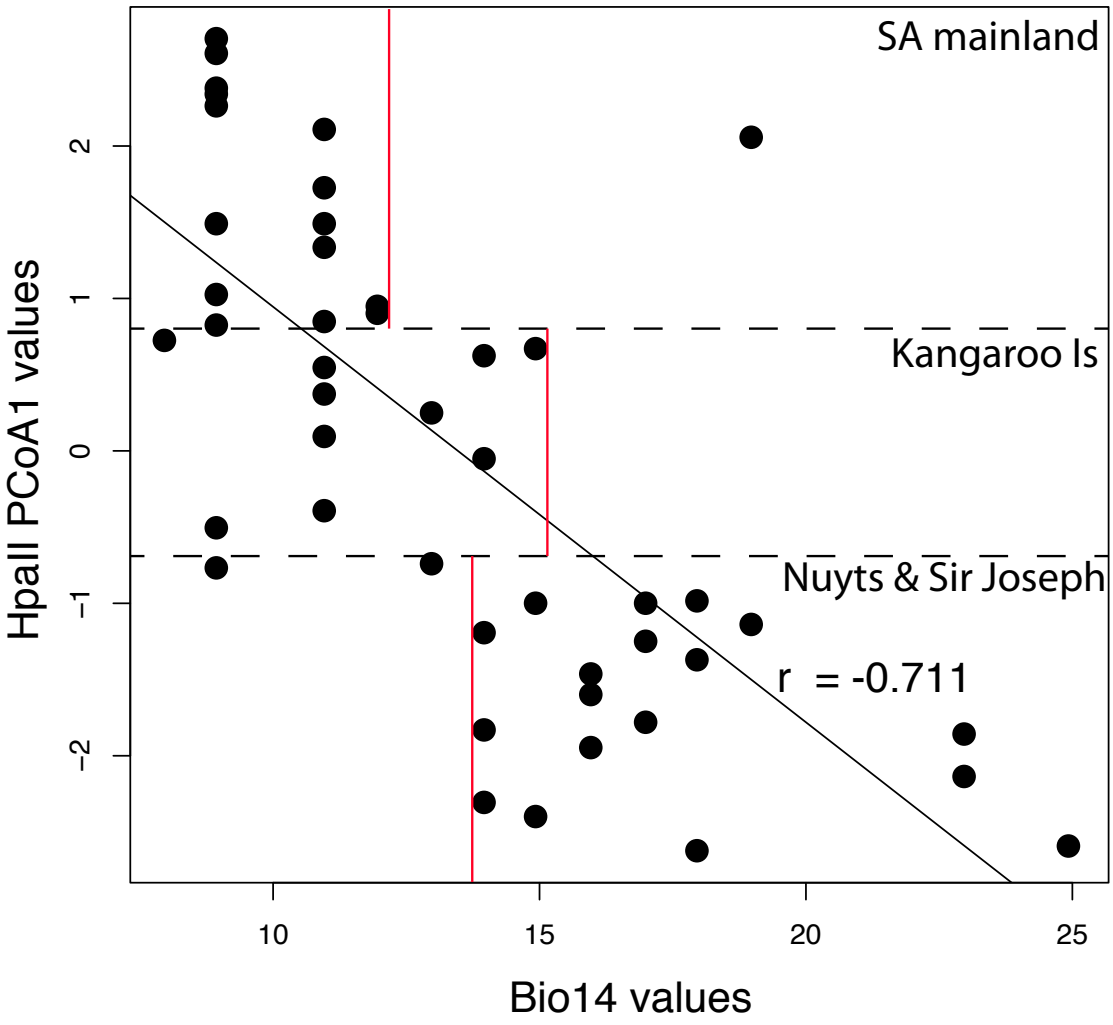


Figure 18. Scatterplot of raw *Hpa*II PCoA1 values vs. Bio14 (precipitation of the driest month) values for Tiger Snakes from South Australian (SA) locations. The horizontal dashed lines roughly separate out the SA mainland Tiger Snakes, from those on Kangaroo Island, and those on islands in the Nuyts Archipelago and Sir Joseph Banks Island group. The SA mainland samples generally experience lower precipitation levels in the driest month (< ~12mm) compared to Tiger Snakes on the Nuyts Archipelago and Sir Joseph Banks islands (> ~12mm), which appears to be related to their genetic/epigenetic profiles (*Hpa*II PCoA1 values). The Pearson correlation coefficient (r = -0.711) shows a strong negative linear correlation.

497

Table 1. Oligonucleotide Sequences used for MSAP

Oligo Name	Step	Sequence
*Hpa*II/*Msp*I Adapter (F)	Restriction/ Ligation	5' GACGATGAGTCCTGAG 3'
*Hpa*II/*Msp*I Adapter (R)		3' CGCTCAGGACTCAT 5'
*EcoR*I Adapter (F)		5' CTCGTAGACTGCGTACC 3'
*EcoR*I Adapter (R)		3'AATTGGTACGCAGTCTAC 3'
*Mse*I Adapter (F)		
*Mse*I Adapter (R)		
Pre-Selective Primer *Hpa*II/*Msp*I	Pre-amplification	5' GATGAGTCCTGAGCGGC 3'
Pre-Selective Primer *EcoR*I		5' GACTGCGTACCAATTCA 3'
Pre-Selective Primer *Mse*I		
*Hpa*II Selective Primer	Selective amplification	5' GATGAGTCCTGAGCGGCCA 3'
*EcoR*I Selective Primer		
*EcoR*I Selective Primer		5' GACTGCGTACCAATTCATG

3’

*Mse*I Selective Primer

498

499

500 Table 2. Each type of methylation as a percentage of fragments genotyped for the SA
501 populations.

Island	CHG %	CG %	Methylation % (CHG + CG)
Continent (n=16)	10	13	22
Goat_Island (n=3)	17	10	26
Franklin Island (n=6)	11	13	24
St Peter Island (n=2)	12	14	26
Hareby Island (n=4)	14	13	27
Reevesby Island (n=5)	14	13	28
Roxby Island (n=2)	13	17	30

502

503 Table 3. Shannon Diversity indices used to estimate the within-population epigenetic
504 diversity (H_{pop}).

	SA mainland	Nuyts Archipelago	Sir Joseph Islands	Kangaroo Island	Species-level
Shannon diversity index (CHG)	2.8188	2.6003	2.5536	2.0440	3.9222
Shannon diversity index (CG)	2.6625	2.5764	2.5584	2.0425	3.8699

505

506

507

508 Table 4. Measures of genetic distance for the AFLP data between the South
509 Australian populations, with values below the diagonal pairwise representing Nei's
510 genetic distance and above the diagonal are pairwise PhiPT (asterisk shows those
511 values significant at the 5% level after 9999 permutations).

	KI (n=7)	Mainland (n=17)	Nuyts (n=12)	Sir Joseph (n=11)
KI	-	0.024	0.124 *	0.080 *
Mainland	0.034	-	0.166 *	0.090 *
Nuyts	0.061	0.072	-	0.041
Sir Joseph	0.050	0.047	0.031	-

512

513 Table 5. The coefficient of epigenetic differentiation was computed as Gst = (Hsp –
514 Hpop)/Hsp where Hsp is the within-species epigenetic diversity.

Gst	Mainland	Island
CHG	0.281311102	0.100645628
CG	0.311996072	0.091400959

515

516

517 Table 6. Measures of epigenetic distance for the *Hpa*II data between the South
518 Australian populations, with values below the diagonal pairwise representing Nei's
519 genetic distance and above the diagonal are pairwise PhiPT (asterisk shows those
520 values significant at the 5% level after 9999 permutations).

	KI (n=5)	Mainland (n=17)	Nuyts (n=12)	Sir Joseph (n=7)
KI	-	0.030 *	0.087 *	0.114 *
Mainland	0.036	-	0.108 *	0.120 *
Nuyts	0.051	0.049	-	0.113 *
Sir Joseph	0.056	0.051	0.046	-

521

522 Table 7. Measures of epigenetic distance for the *Msp*I data between the populations,
523 with values below the diagonal pairwise representing Nei's genetic distance and
524 above the diagonal are pairwise PhiPT (asterisk shows those values significant at the
525 5% level).

	KI (n=7)	Mainland (n=17)	Nuyts (n=12)	Sir Joseph (n=11)
KI	-	0.097 *	0.090 *	0.083 *
Mainland	0.055	-	0.067 *	0.106 *
Nuyts	0.050	0.037	-	0.061 *
Sir Joseph	0.047	0.050	0.035	-

526

527

528 Table 8. Correlation between genetic/epigenetic distance (*Hpa*II PCoA1 pairwise
529 distance matrix) and bioclimatic variables (pairwise distance matrix) for each SA
530 sample.

Variable	Mantel test p-value	Pearson correlation coefficient	Mainland values	Nuyts Archipelago & Sir Joseph Banks Island values
Bio1: Annual mean temp (°C)	**0.001****	**0.652**	**> 15.8**	**< 16.3**
Bio2: Mean Diurnal Range	0.779	0.083		
Bio3: Isothermality (BIO2/BIO7) (* 100)	0.115	0.261		
Bio4: Temperature Seasonality	0.574	-0.015		
Bio5: Max Temperature of Warmest Month	0.578	0.174		
Bio6: Min Temperature of Coldest Month	0.068	0.215		
Bio7: Temperature Annual Range (BIO5-BIO6)	0.737	0.018		
Bio8: Mean Temperature of Wettest Quarter	0.001**	0.458	> 12.0	< 13.5
Bio9: Mean Temperature of Driest Quarter	0.002*	0.469		
Bio10: Mean Temperature of Warmest Quarter	0.002*	0.478		
Bio11: Mean Temperature of Coldest Quarter	0.001**	0.598	> 12.0	< 12.2
Bio12: Annual Precipitation (mm)	0.001**	-0.506	< 540	280—800
Bio13: Precipitation of Wettest Month	0.008	-0.371		
Bio14: Precipitation of Driest Month	**0.001****	**-0.711**	**< 12**	**> 14**
Bio15: Precipitation Seasonality	0.111	0.186		
Bio16: Precipitation of Wettest Quarter	0.008	-0.405		
Bio17: Precipitation of Driest Quarter (mm)	0.001**	-0.691	< 46	> 47

Bio18: Precipitation of Warmest Quarter	0.001**	-0.677	< 47	> 48
Bio19: Precipitation of Coldest Quarter	0.001**	-0.411	125—240	80—330

531 One asterisk (*) signifies Bonferroni corrected p-values less than 5% significance
532 level (0.0026), with two asterisks (**) representing Bonferroni corrected p-values less
533 than 2.5% significance level (0.0013).
534