Final report

GENETIC SURVEY OF THE PILBARA OLIVE PYTHON

(*Liasis olivaceaus barroni*)

David Pearson[1], Peter Spencer[2] Mia Hillyer[2] and Ric A. How[3]

[1]Science Division,
Department of Parks and Wildlife
PO Box 51, Wannerooo, WA 6946

[2]School of Veterinary and Life Sciences,
Murdoch University
90 South St, Murdoch, WA 6150

[3]Department of Terrestrial Zoology (Vertebrates),
Western Australian Museum,
49 Kew St, Welshpool, WA 6986

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Summary

- The study used genetic information to investigate differences between and within populations of olive pythons in the Pilbara. This information was compared with genetic profiles from olive pythons form the Kimberley and carpet pythons.

- Genetic variation was examined at eight nuclear genes (microsatellite) from 47 individual olive pythons.

- Genetic analyses of nuclear markers show that the Pilbara olive python contains low levels of diversity, compared with its Kimberley counterpart.

- The Pilbara population also had a low effective population size, but showed no signatures of a genetic bottleneck as a result of a population crash.

- Nuclear DNA markers identified two distinct olive python populations. One in the Pilbara and the other in the Kimberley.

- Mitochondrial analysis at three diagnostic regions showed two distinct clades representing Pilbara and Kimberley olive pythons, exclusively, consistent with results from nuclear markers.

- Overall olive pythons appear to have two Evolutionary Significant Units. The Pilbara unit appear to be less genetically diverse than Kimberley one and shows little phylogeographic structure within the Pilbara.

- There is sufficient evidence from the data that the taxonomy of the two groups should be subject to a re-appraisal, the Kimberley and Pilbara Olive pythons sufficiently different to be considered as different species.

- The genetic markers are ideal as a tool in wildlife trafficking and forensic investigations into both smuggling and population identification.

1. Introduction

The Pilbara olive python, *Liasis olivaceus barroni*, is a large snake restricted to the Pilbara region and the northern-most section of the Ashburton region of Western Australia. It is a large snake (up to 6.5 m in length, Shine 1981) that has avoided close scrutiny, with few museum specimens or published accounts of this species. Very little is known about its biology or its distribution in the Pilbara. The Pilbara olive python is separated from more northern tropical populations of olive pythons by vast expanses of the Great Sandy Desert that are inhospitable for such snakes (Pearson 1993, 2006).

Current knowledge suggests that Pilbara olive pythons occur from Yinnietharra Station in the northern Ashburton region north to near Port Hedland in the Pilbara. They extend as far east as the Ragged Hills and the drainage of the Oakover River, while an isolated population occurs on Dolphin Island and the former Burrup Island, the latter now joined to the mainland by a causeway.

The Pilbara olive python is listed under the Commonwealth Environment Protection and Biodiversity Conservation Act (1999) as threatened fauna ("Vulnerable") and on Schedule 1 of the Western Australian Wildlife Conservation Act (1950) as "Fauna that is rare or likely to become extinct". The python is well known to Aboriginal people of the Pilbara ("bargumyji" to the Yindjibarndi and "palkunyji" or "parkunarra" to the Kurrama people) and was once an important food. It remains an important spirit figure in explanations of the formation of Pilbara landscapes, especially rivers and waterholes (Pearson 2007).

The earliest European record of this python appears to be an observation by the explorer Ernest Giles in May 1876. In the upper reaches of the Ashburton River, he noted that one of his companions:

> "shot a very large snake; it was nearly nine feet long, was a foot round the girth and weighed nearly fifty pounds. It was a perfect monster for Australia. If we had been without food what a godsend it would have been."

Pilbara olive python specimens have been sent to the Western Australian Museum infrequently. Four are noted on the register prior to 1960; six more were collected in the 1960s; three in both the 1970s and 1980s; but just one in the 1990s, principally due to its listing as threatened fauna. In recent years, more have been sent to the Museum as new areas are opened up by resource projects.

In 1981, Laurie Smith of the Western Australian Museum described the Pilbara olive python on the basis of just eight specimens then available in the collection. He distinguished it from the northern populations of *Liasis olivaceus olivaceus* on the basis of having fewer midbody scale rows (58-63 versus 61-72) and a greater number of ventral scales (374-411 versus 355-377). Smith (1981) named what he considered a subspecies of the more widespread olive python, "barroni", in honour of Greg Barron for his services to Western Australian herpetology.

Since that time there have been suggestions that the Pilbara olive python may deserve recognition as a full species. More recently, it has been suggested that some populations within the Pilbara may be genetically different due to their isolation. There is an urgent need to resolve the taxonomic uncertainty surrounding this large python and its various populations, so that its conservation can be adequately considered and managed. It is a top-order predator of mammals, reptiles and birds and uses rocky gorges and waterways for hunting and shelter. Consequently, the Pilbara olive python is potentially sensitive to disturbances to its' habitat and prey resources with increasing infrastructure and mineral resource development throughout the Pilbara. There is now compelling evidence that inbreeding and loss of genetic diversity compromise the viability of wild populations (Frankham et al. 2009). There is also an increasing awareness of

the need to consider genetic issues in the management of threatened species and that ignoring genetic factors may lead to inappropriate management strategies. This is of considerable relevance to the Pilbara olive python because of the lack of genetic information about the species.

1. 1 Study aims

The aim of this project is to clarify the genetic status of the Pilbara olive python to allow more informed management of its populations.

Specifically, is the Pilbara olive python:

1. A species in its own right, or a subspecies of the widespread Northern olive python?

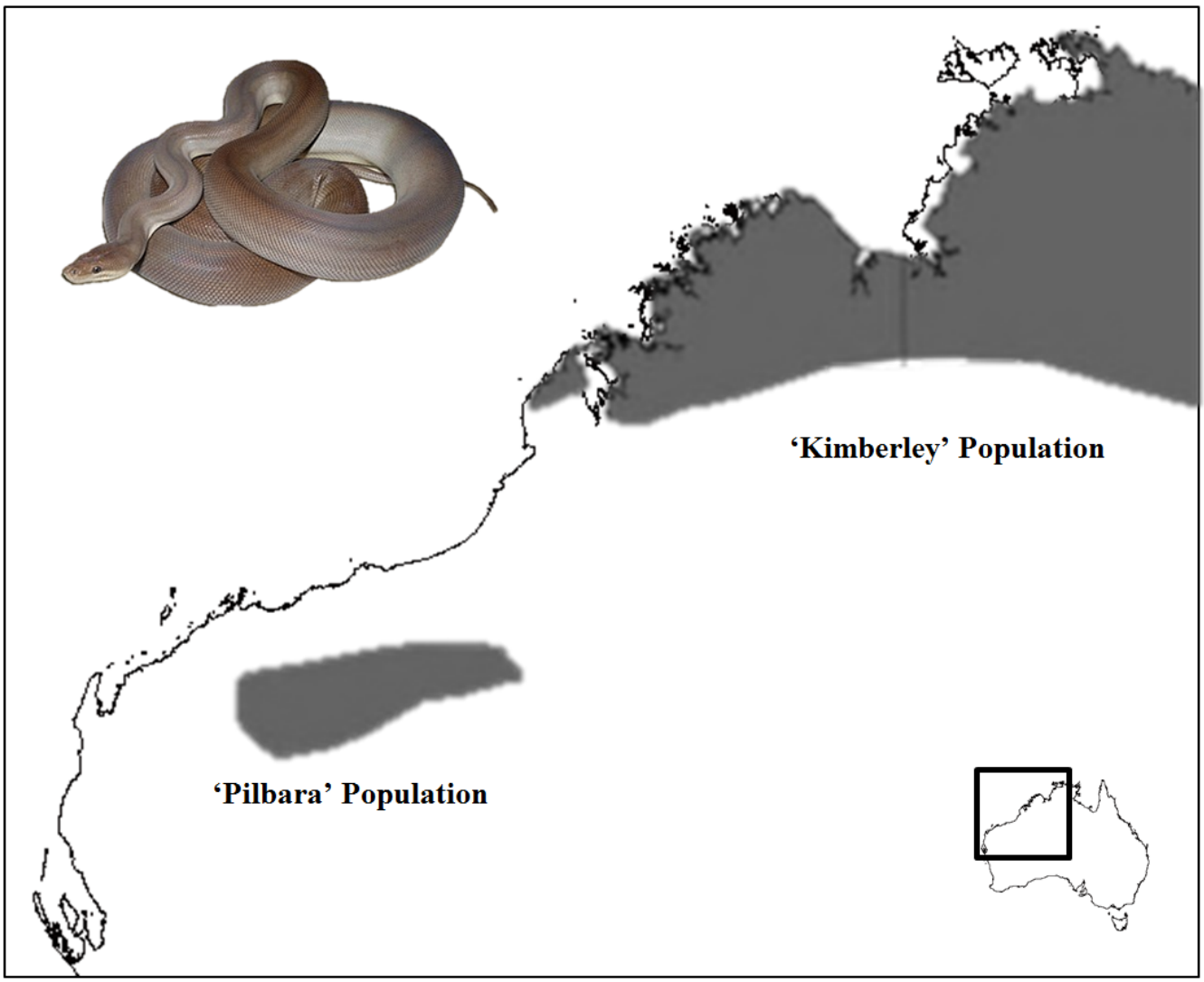
2. Exhibit any evidence of genetically distinct management units within the Pilbara?

3. As genetically diverse as pythons from the Kimberley region?

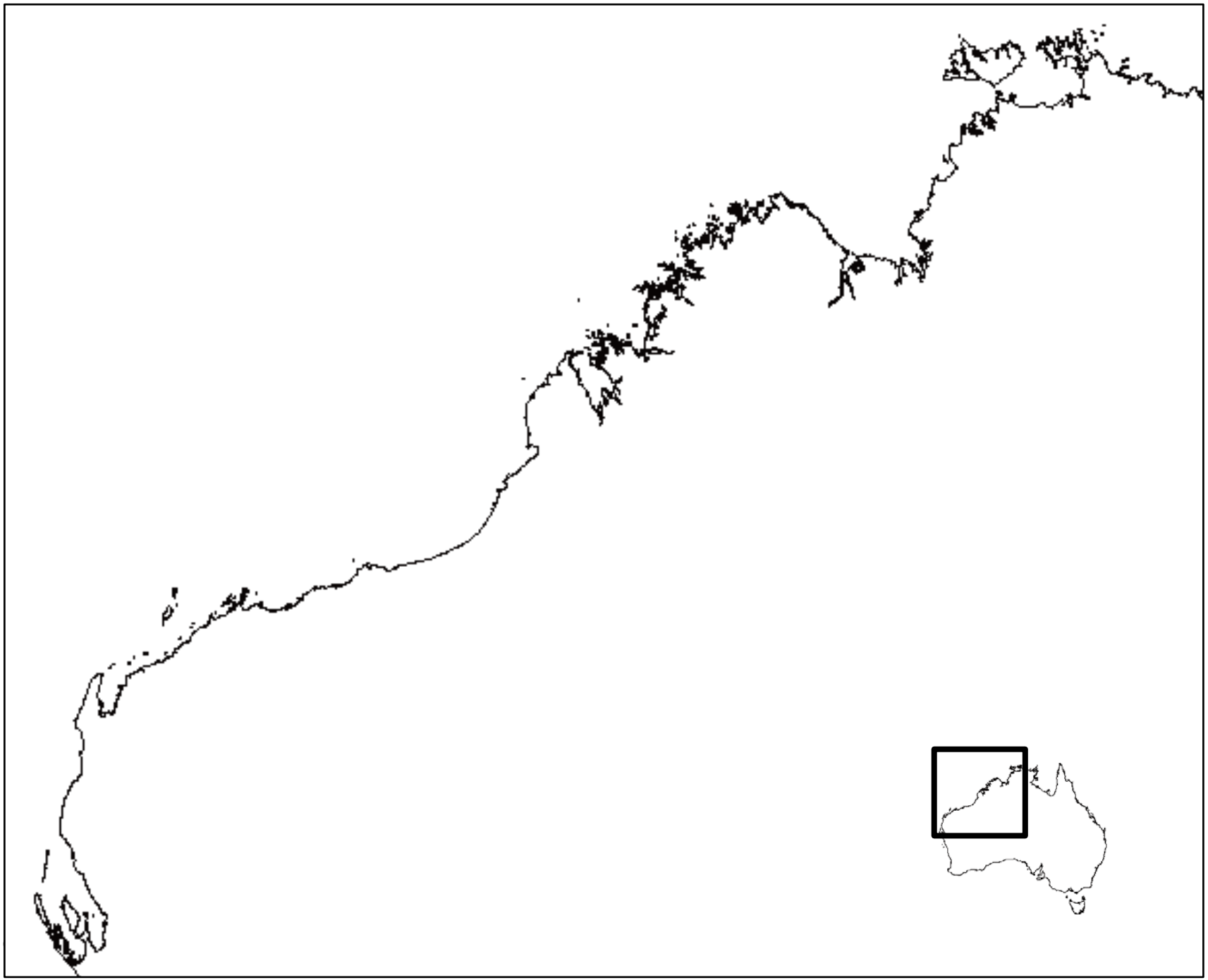
4. And lastly, are the genetic tools useful for identifying individuals and the origin of pythons in cases of the illegal wildlife trade?

2. Samples and Methods (Laboratory and Analyses)

To date, a total of 47 olive pythons were available for analysis. Of those, 25 were



from the Pilbara and 22 from the Kimberley. A large number of the Kimberley samples (52%) were from around Kununurra. Thirty two Carpet Pythons were included as an 'out group' to the olive pythons, and served as a reference group.



Molecular Methods

Nuclear microsatellite amplification and analysis

We amplified eight microsatellite loci as described in Rawlings (2008) from sampling locations in the Pilbara and Kimberley. Briefly, PCRs were carried out in a total volume of 30 µl with ~100 ng DNA, 1X PCR buffer, 400 µM of dNTPs, 2mM $MgCl_2$, 0.2 µM of each primer & 0.825 U *Taq*. Size was determined by co-running a Genescan500 standard (Applied Biosystems, Melbourne). Fluorescently-labelled DNA fragments were separated using an ABI373*xl* capillary sequencer (Applied Biosystems) and scored manually with the aid of GENEMARKER software (v1.5, Soft Genetics). Data was checked for input errors and duplicate genotypes using the Excel Microsatellite Toolkit add-in (Park 2001). Deviations from Hardy-Weinberg equilibrium, linkage disequilibria and the presence of null alleles were tested using HW-QUICKCHECK (Kalinowski 2006), GENEPOP, (Raymond & Rousset 1995) and MICROCHECKER (van Oosterhout et al. 2004), respectively.

Population structure was inferred using STRUCTURE v 2.3 (Pritchard et al. 2000), based on repeated simulations from K=1 to K=10 inferred populations, using 10^6 iterations of a Markov Chain Monte Carlo (MCMC) simulation and a burn-in period of 50,000 iterations. The optimum values of *K* was determined using an ad hoc statistic (ΔK) based on the rate of change in the log probability of data between successive *K* values as described by Evanno et al. (2005). The level of genetic differentiation among populations was determined by estimating F_{ST} (denoted as θ, Weir & Cockerham 1984), using Fisher's exact tests for genetic differentiation from allele frequencies (Goudet et al. 1996) as well as R_{ST} and Rho (FSTAT 2.9.3; Goudet 1995 and GENALEX 6.3). Descriptive statistics were calculated using GenAlEx v 6.3 (Peakall and Smouse 2006) and included the number of alleles (N_A) and effective alleles per locus (N_E), as well as observed and expected heterozygosities.

Detecting a change in the demographic history used a method developed by Luikart et al. (1998) and tested for distortion of allele frequency distributions (Luikart and Cornuet 1998) as a result of rarer alleles being more likely to be lost during a bottleneck than common alleles. This test for a genetic bottleneck is more appropriate for populations that has been reduced very recently, with less severity and the pre-bottleneck value of θ was small (see Williamson-Natesan 2005). The bottleneck results in an excess of heterozygosity under a stepwise mutation model. To detect this, we use the program BOTTLENECK 1.2 (Piry et al. 1999). Due to the relatively small number of loci analysed (n = 8), a Wilcoxon sign-rank test was estimated, as recommended by Piry et al. (1999). A mixed model of microsatellite mutation was assumed, with single step mutations assumed to account for 90% of all mutation events, and a variance among multiple steps of 12, as suggested by Piry et al. (1999).

Mitochondrial amplification and analysis

We also amplified control region (or *Dloop*), the cytochrome b (*cyt b*) gene and the cytochrome oxidase I (*COXI*) gene of the mitochondria. If there is variation and differences in the sequences, then this region is diagnostic of sub/specific differences. A small sample of carpet pythons and a water python were included as the outgroup(s). The outgroup is a taxon outside the group of interest. All the members of the group of interest (olive pythons) are more closely related to each other than they are to the outgroup (carpet/water python). Hence, the outgroup stems from the base of the tree. An outgroup can give you a sense of where on the bigger tree of life the main group of organisms falls.

Fewer specimens were sequenced at the cytochrome oxidase gene (COXI) as it would be unlikely to reveal fine-scale patterns. The COXI gene is often diagnostic in identifying species levels questions, and known as a useful ‘DNA barcoding’ marker.

3. Results

Genetic diversity and population genetic 'health'

A total of 25 Pilbara and 22 Kimberley olive python, along with 32 Carpet Python, samples were successfully scored at eight highly variable microsatellite loci. All the sample groups were polymorphic at all loci with moderate variation, containing between 1 and 23 alleles per locus (7.96 ± 1.31) with heterozygosity (H_E) ranging from 41 to 73% (mean = 0.73 ± 0.11; Table 1).

Table 1. Measures of microsatellite variability of the three sampled populations of pythons. *n*, number of individuals genotypes. Values given as a mean ± S.E. (standard error).

Inferred population	n	Number of alleles	Effective number of alleles	Expected heterozygosity	Observed heterozygosity
Pilbara olive python	25	5.37 ± 1.75	3.24 ± 0.95	0.41 ± 0.15	0.36 ± 0.14
Kimberley olive python	22	8.63 ± 2.39	5.76 ± 1.58	0.70 ± 0.09	0.64 ±0.09
Carpet Python	32	9.88 ± 2.57	5.42 ± 1.26	0.73 ± 0.11	0.48 ±0.11

The olive pythons showed fixation values (*F*) that suggest the populations show random mating (Table 2). Estimates of the effective population size (N_e) showed a very low estimate (N_e =5) from the Pilbara, compared with the Kimberley sample (N_e =160). However, this may simply reflect the localised sampling effort.

Table 2 No genetic bottlenecks were found in any python populations. ; Fixation index values around 0 suggest mating is random, +1 highly inbreed;

Inferred population	Fixation Index (*F*)	Genetic bottleneck	Significance (*P*-value)
Pilbara olive python	0.09 ± 0.05	No	0.9375
Kimberley olive python	0.05 ± 0.08	No	0.4219
Carpet Python	0.33 ± 0.01	No	0.4010

How unique is the Pilbara population? Differentiation among and within populations?

All genotyped individuals clustered with their source population (Table 3). This can be clearly seen by the close clustering of the Pilbara samples in Fig. 1. We detected two discrete olive python population clusters (K=2; Fig. 1) in Western Australia, one from the Pilbara region and another being the genetically distinct Kimberley olive python. All individuals assigned correctly to their sampled population (Table 3), allowing great certainty in determining provenance of an unknown sample

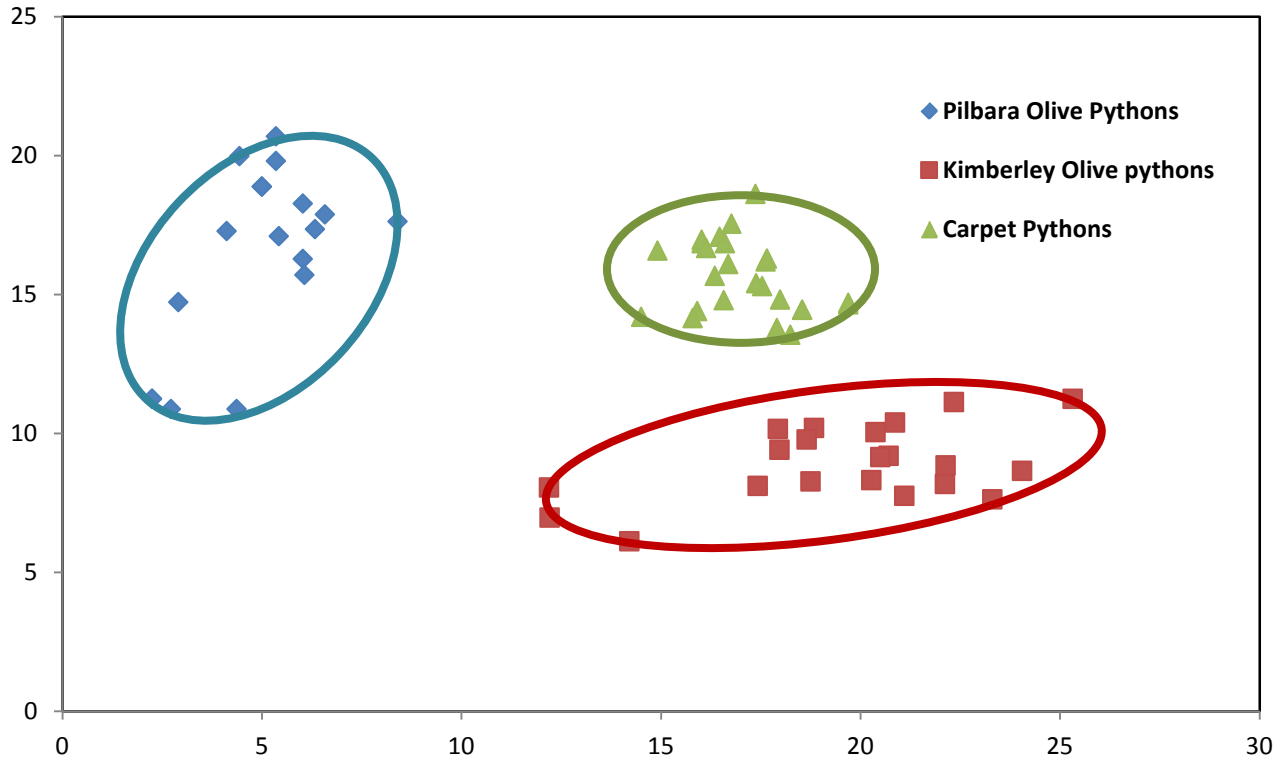


Figure 1 Assignment of the 25 Pilbara and 22 Kimberley olive pythons to sampling locations based on eight nuclear microsatellite loci. Individuals are encompassed into sampled populations by coloured circular lines. The axes are arbitrary. The analysis is referred to as an Assignment Test (generated using the program GenAlEx) shows three distinct populations, corresponding to the two olive python clusters and a cluster from the Carpet Python. Proximity does not infer relatedness (i.e. Carpet Pythons are not necessarily genetically closer to Kimberley than Pilbara olive pythons).

Table 3. The number of pythons that were assigned to their own population (self-population) or clustered with another population (Other pop).

Population	Self-Population	Other Pop	% assigned to another population
Pilbara olive pythons	18	0	None
Kimberley olive pythons	22	0	None
Carpet Pythons	25	0	None
Total (Percentage)	65 (100 %)	0 (0 %)	

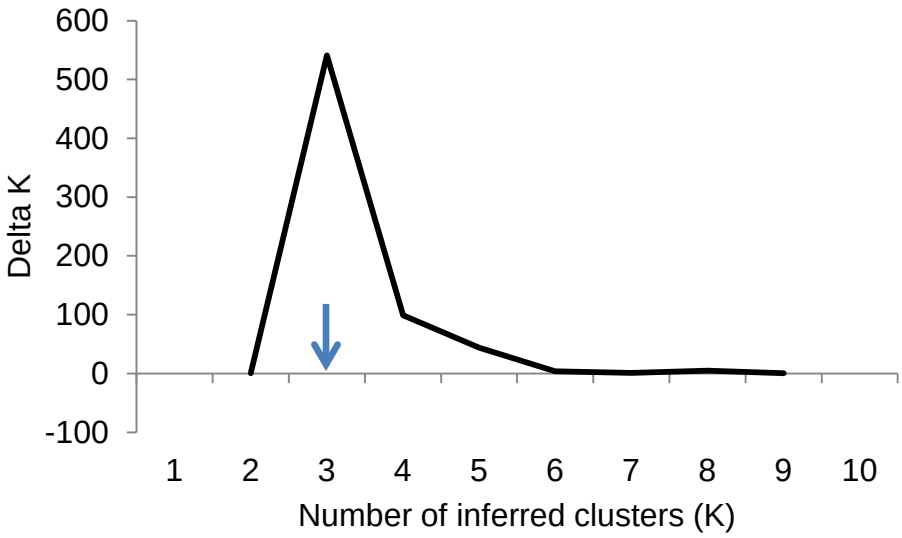


Figure 2 The rate of change in the STRUCTURE likelihood function (Delta or ΔK values) corrected for larger variance with increasing value of K) as a function of the number of inferred clusters (K). The figure shows that the data is best explained by three population clusters (that correspond to the (i) Pilbara, (ii) Kimberley olive pythons and the (iii) carpet python).

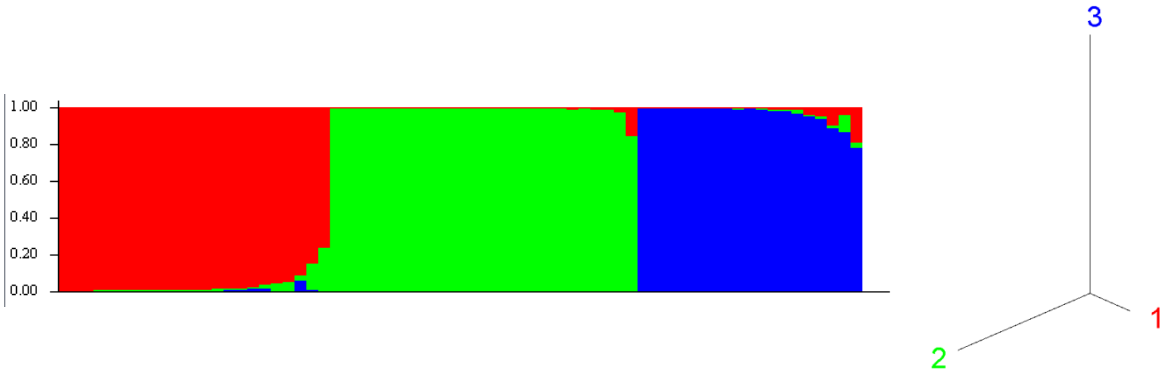


Figure 3 Bayesian population structure analyses. Bayesian assignment of the Pilbara (red), Kimberley (green) olive python and the carpet python (blue), based on 8 nuclear microsatellite loci, assuming a population number of K = 3. Individuals are along the x-axis. The y-axis denotes the cumulative posterior probability of an individual's placement in particular population(s). Not surprisingly, individual clusters show that the olive pythons are more closely related than the carpet python (figure to right).

Global (pooled) estimates of F_{ST} (0.292 ± 0.077) between the three python groups identified were similar and indicated moderate-high levels of genetic differentiation amongst these populations, and a very large difference between the Olive and Carpet Python groups (Table 4). Pair-wise F_{ST} values (Table 4) indicated moderate to high levels of differentiation between all pairs of populations (i.e. al values >0.1)

Table 4. Pairwise F_{ST} estimates of population differentiation among python populations, based upon the observed genotypes that were estimated from eight microsatellite loci. Values above 0.2 indicate a high degree of genetic differentiation.

	Pilbara	Kimberley	Carpet Python
Pilbara	-		
Kimberley	0.165	-	
Carpet Python	0.350	0.191	-

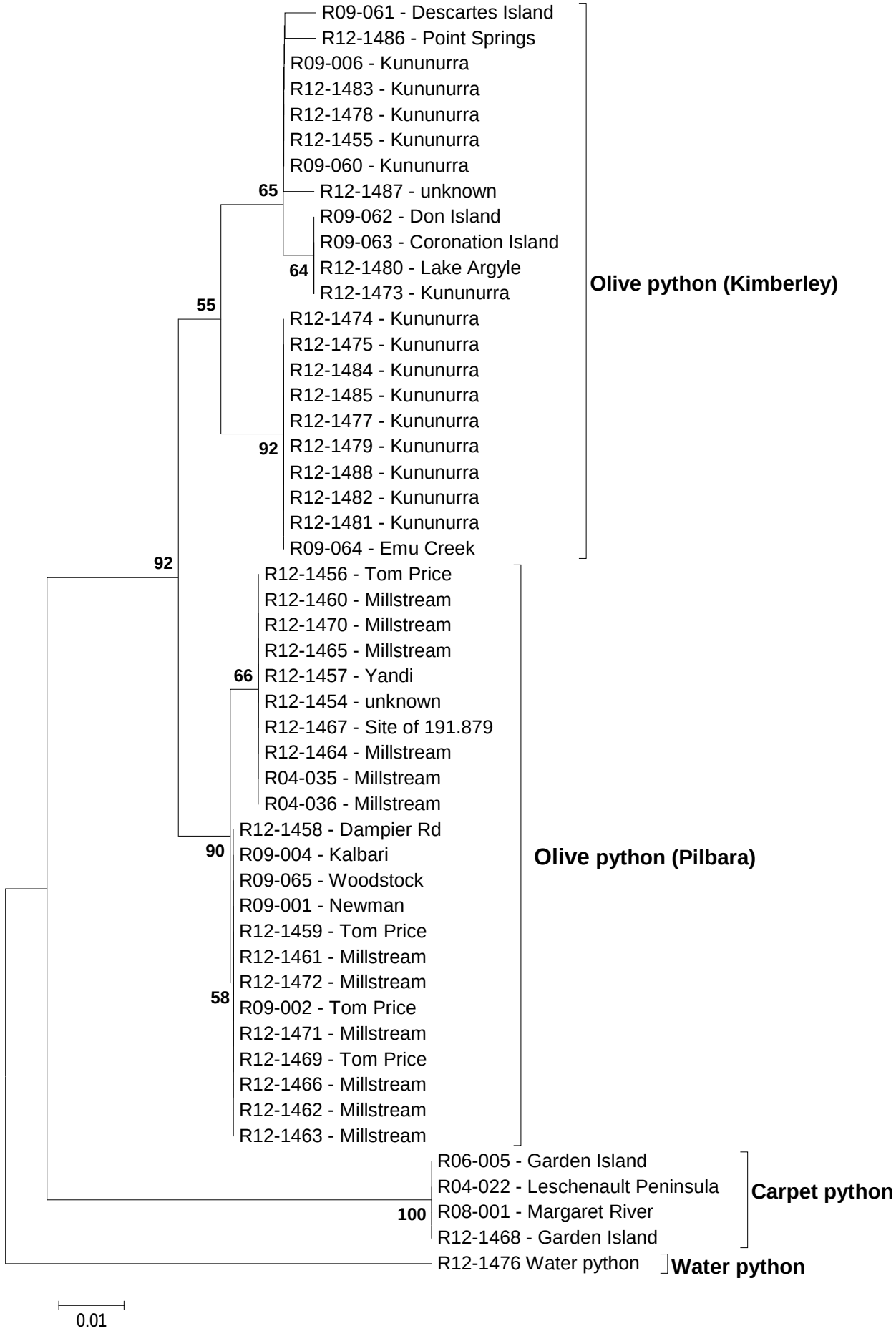


Fig 4. Basic neighbour-joining tree generated from sequences from the 1029 base pairs of the cytochrome b region. Complete deletion of missing sites

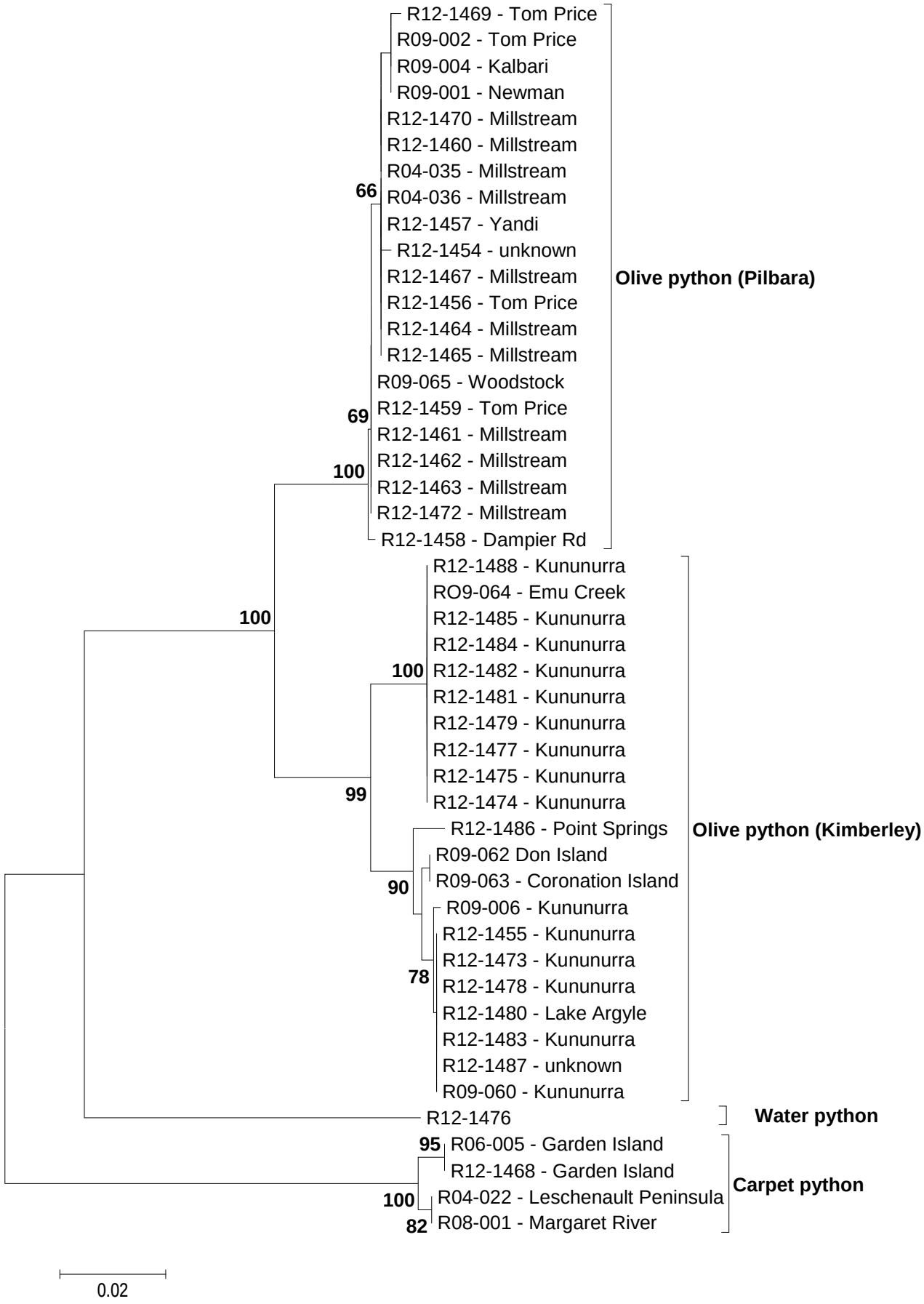


Fig 5. Neighbor-joining tree generated from sequences from the 1029 base pairs of the control (D-Loop) region. Analysed using the complete deletion of missing sites.

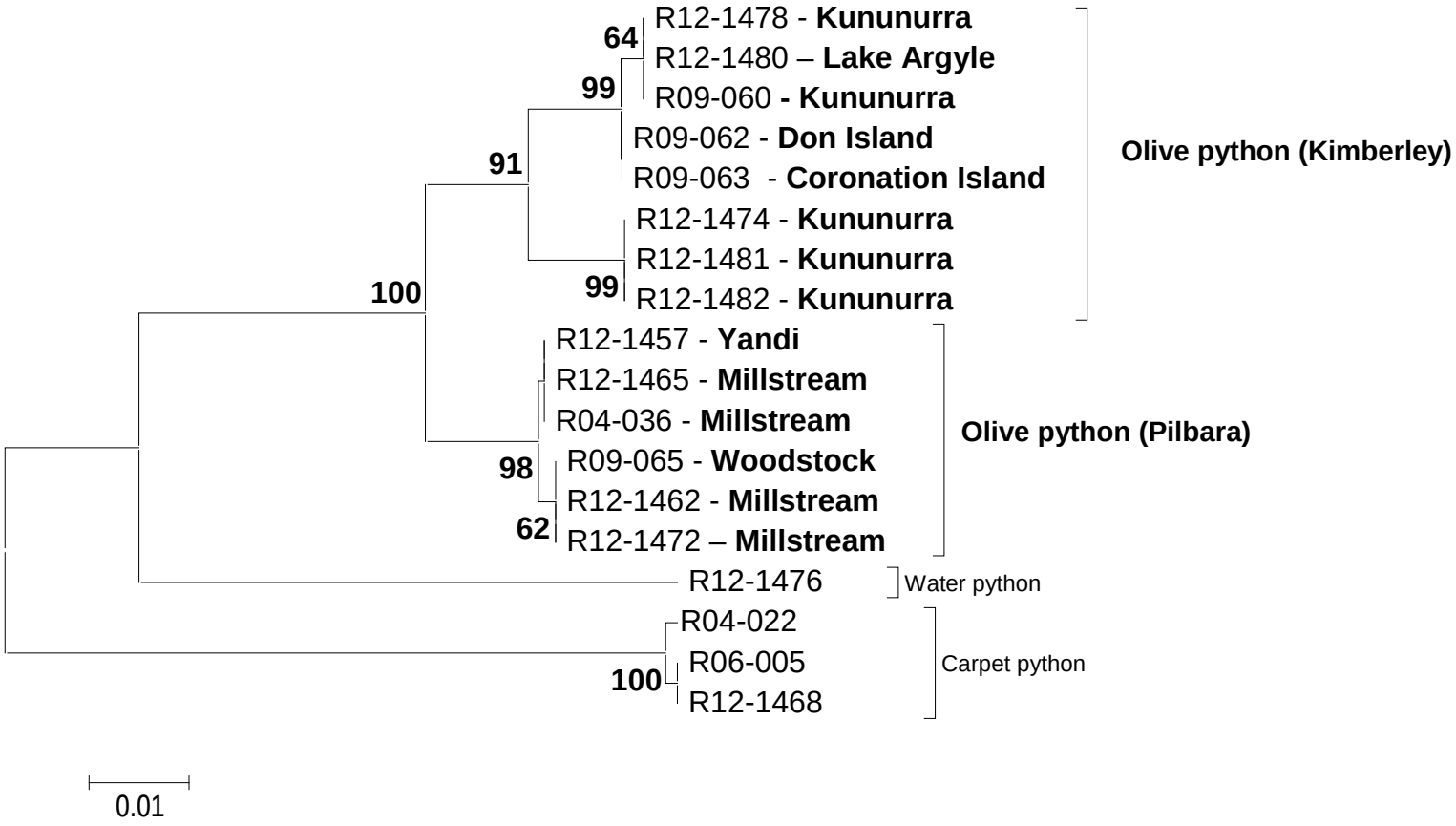


Figure 6 neighbour-joining tree generated from sequences from the 1029 base pairs of the COX1 (cytochrome oxidase) region. Analysed using the deletion of all incomplete sequences from sites. The optimal tree with the sum of branch length = 0.2095 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [3] and are in the units of the number of base substitutions per site. The analysis involved 18 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 432 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [4].

Analysis of the three mitochondrial genes at the cytochrome b (*cytb*) region, control region (or *Dloop*) and cytochrome oxidase (*COXI*) identified a clear distinction between the three groups of pythons. There were two distinct olive python clades in the Pilbara (Fig 4), however interpreting any other 'variation' or diversity within the population was not useful, as individuals from the same sampling site (Kununurra) were found in both clades. Along the same lines, it was not uncommon to identify individuals from islands and mainland sites grouping together (e,g, Don Island & Coronation Island; Fig 4). Overall, the analysis of the mitochondrial genes reiterated the finding from the nuclear markers, namely that there are two distinct forms of olive pythons, one from the Pilbara and the other from the Kimberley region.

Mitochondrial markers were extremely diagnostic for provenance of a sample, but not suited for individual identification. However, the use of nuclear microsatellite markers allowed identification of both the origin of the sample (either Pilbara or Kimberley) and it also generated a highly discriminatory DNA profile. These markers were suitable for identification of an individual and sufficiently discriminatory to exclude other individuals with high certainty.

Forensic profiling: How unique are individuals and is it possible to identify where they came from?

An important application of this technology is to be able to identify a python, and where that python may have originated. An estimation of how unique each python was showed that by genotyping an individual at four or more loci enabled a confidence of >99.9% to be estimated on correct identification (Fig. 7). Considering the case of a python genotyped using a combination of eight microsatellite loci showed that it would be expected that the probability of two randomly sampled snakes sharing the same genotype as 1 in 82,000 Pilbara olive pythons, 1 in 462 million Kimberley olive pythons (and 1 in 394 million carpet pythons; Table 5).

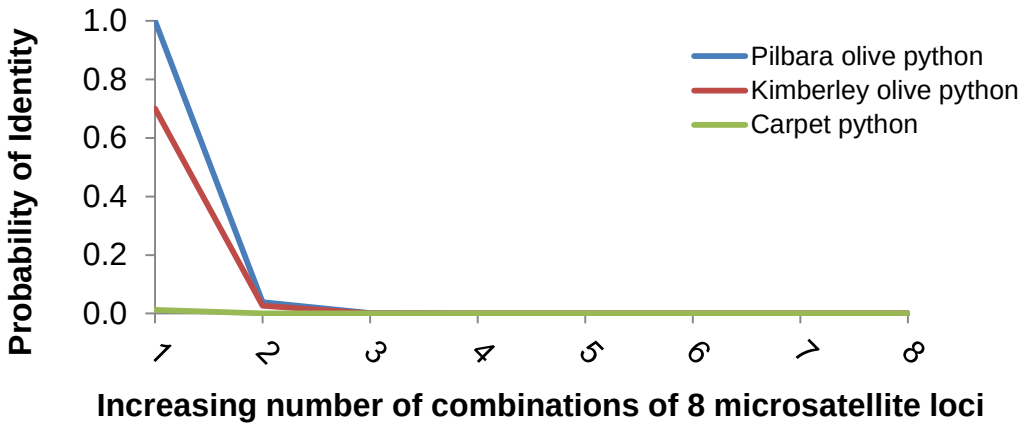


Figure 7. Using a combination of microsatellite markers identified that with more than four loci, the probability that two randomly chosen pythons will share exactly the same genotype at those loci would be <0.001%

Table 5. A range of statistics that demonstrate how 'individual' a DNA profile in pythons should be.

Population /species	Probability of identity	Exclusion probability	Expected No. individuals with the same multilocus genotype
Pilbara olive python	1 in 81,600	1.2×10^{-5}	3×10^{-4}
Kimberley olive python	1 in 462,100,000	2.2×10^{-9}	4.8×10^{-8}
Carpet python	1 in 394,500,000	2.5×10^{-9}	8.1×10^{-8}

4. Discussion

How unique are Pilbara and Kimberley Python populations and is the Pilbara olive python a species in its own right, or a subspecies of the widespread Northern olive python?

Genetically, the Pilbara and Kimberley populations of olive pythons are genetically dissimilar. This conclusion was supported by information from both nuclear and mitochondrial markers. Using genetic information alone, the two should be considered different species, and as such, a taxonomic revision is warranted.

How important is the Pilbara python in term of genetic diversity, and is there evidence of genetic structuring in the Pilbara?

The Pilbara olive python is a unique evolutionary significant unit but in terms of diversity, olive pythons from the Pilbara contain substantially less diversity than pythons from the Kimberley. This finding is consistent for both mitochondria genes and nuclear microsatellites. Despite a small sample, there appears to be little structuring within pythons from the Pilbara.

Are Pilbara olive pythons as genetically diverse as their Kimberley counter parts?

The Pilbara olive pythons contain only moderate levels of genetic diversity, compared with olive pythons form the Kimberley. There appears to be a trend for the Pilbara fauna to have generally lower diversity (if there are a comparative group to) in comparison to the Kimberley, for example the same pattern is found with northern quolls. This may reflect the more insular structure of the Pilbara, where movement may be more restricted.

The illegal trade in **wildlife: Are the genetic tools useful for identifying (i) individuals and (ii) the origin of pythons**

This work illustrates the value of nuclear microsatellite and mitochondrial markers in identifying;

- wildlife trafficking
- illegal take and smuggling as well as,
- the potential to verify parentage of captive stock.

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