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OzPythonPlex: An optimised forensic STR multiplex assay set for the Australasian carpet python (*Morelia spilota*)

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Highlights

- Three multiplex assays each with 11 hypervariable STR loci have been developed from the genome of the carpet python
- Multiplex assays are optimised for peak balance and low starting template of 1 ng, yielding a complete 24 locus STR profile from 3 ng DNA template
- Three locus redundancy has been included across assays as a quality assurance measure
- A comprehensive allelic ladder has been created for each multiplex assay
- A mini database based on the 24 loci has been generated to allow standard association probabilities to be performed

Abstract

Reptile species, and in particular snakes, are protected by national and international agreements yet are commonly handled illegally. To aid in the enforcement of such legislation, we report on the development of three 11-plex assays from the genome of the carpet python to type 24 loci of tetra-nucleotide and penta-nucleotide repeat motifs (pure, compound and complex included). The loci ranged in size between 70 and 550 bp. Seventeen of the loci are newly characterised with the inclusion of seven previously developed loci to facilitate cross-comparison with previous carpet python genotyping studies. Assays were optimised in accordance with human forensic profiling kits using one nanogram template DNA. Three loci are included in all three of the multiplex reactions as quality assurance markers, to ensure sample identity and genotyping accuracy is maintained across the three profiling assays. Allelic ladders have been developed for the three assays to ensure consistent and precise allele designation. A DNA reference database of allele frequencies is presented based on 249 samples collected from throughout the species native range. A small number of validation tests are conducted to demonstrate the utility of these multiplex assays. We suggest further appropriate validation tests that should be conducted prior to the application of the multiplex assays in criminal investigations involving carpet pythons.

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Key words: allelic ladder, carpet python, DNA profiling, STR typing, validation, wildlife forensic science

1. Introduction

The extent of the global illegal wildlife trade is attracting increasing attention, evidenced in part by the recent publication of the inaugural United Nations Office on Drugs and Crime (UNODC) World Wildlife Crime report [1]. The growth of interest in wildlife forensic applications from within the forensic science community is also gaining momentum with the inclusion of a Wildlife Forensics Subcommittee in the Biology/DNA Committee of the Organisation of Scientific Area Committees (OSAC) for Forensic Science [2]. Iconic species such as elephant, rhino and tiger are the public face of wildlife crime, yet reptiles constitute the second largest taxonomic class among seizures worldwide [1], although seizures only represent detected, not total crime and reptiles are easily concealed [3,4]. Seizures indicate that reptiles dominate the live-trade international black market [5]. A prime example is the Australasian carpet python (*Morelia spilota*) which, although native to Australia, is highly sought after for legal breeding and trading both within Australia and abroad [6]. However, its unique colour and pattern-variations fetch high prices on the international black market [6], which creates high demand for breeding within and between closely related species to create new morphological varieties. Due to their compact size and low maintenance, carpet pythons have gained popularity as household pets and are commonly traded through pet shops, dedicated reptile expos, and online and often in offline reptile networks spread by word of mouth (H. Dridan, *pers. comm.*). The breeding and keeping of carpet pythons is regulated in Australia through various state and national legislation. Despite the removal of a native python from the wild without a permit being unlawful in Australia under the Commonwealth Environmental Protection and Biodiversity Conservation Act 1999, native populations provide an accessible source of fresh genetic variability to increase the genetic health of a small, continuously inbred captive collection. The increase of wildlife enforcement investigations involving Australian pythons across multiple Australian States has led to a direct request to the authors from enforcement authorities for the development of DNA tools to aid enforcement efforts involving carpet pythons [7].

Due to their high cost of development, the species-specific nuclear Short Tandem Repeat (STR) DNA markers used for individualisation and paternity testing have frequently been developed for species with high commercial returns, such as game animals e.g. deer [8–10] and wild boar [11] and prominent fisheries species e.g. salmon [12] and koi carp [13]. Animals often in conflict with humans

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have also been targeted, including those threatening human safety (e.g. bears [14], leopards [15], wolves [16]) as well as those considered a pest for their impact on human industry (e.g badgers [17,18], foxes [19]). Profiling assays for companion animals have been used as independent evidence in crimes against humans [20–22] and increasingly tests are being developed for wildlife species targeted directly in criminal activities, such as birds of prey [23] and cockatoos [24]. The importance of extensive validation testing for direct forensic application is recognised [22,25,26]. Utility of STR marker systems has been further extended to provide intelligence about illegal poaching 'hotspots' through geographic assignment of poached individuals [27–29]. The application of eight previously characterised STR markers for carpet pythons [30] was found to provide inadequate genetic resolution to answer questions commonly occurring in wildlife investigations [31] and identified the need for development of a larger profiling marker set for this species.

We present herein the development and a limited developmental validation of three novel 11-plex multiplex assays for forensic DNA profiling of the carpet python to assist in criminal investigations. The multiplex assays have been designed and developed following best practices evolved from the development and validation of commercial human forensic multiplex assays, including use of tetra- and penta-nucleotide repeat motifs to minimise stutter artefacts, input DNA template concentration optimised to 1 ng/µL and optimisation of peak balance across a profile to indicate when null alleles are present. All work has been conducted according to the ISFG recommendations regarding the use of non-human (animal) DNA in forensic genetic investigations [32] and following the guidelines of the Scientific Working Group on DNA Analysis Methods [33].

2. Materials & Methods

2.1. Samples

Supplementary Material 1 provides the details of the 304 samples examined in this study. All reference samples were obtained from the Australian Biological Tissue Collection of the South Australian museum. Voucher specimens were included where available. Bredl's python (*Morelia bredli*) were included in this study, as a previous phylogenetic analysis [34] suggests that this species is genetically less divergent than *M. spilota imbricata*. Tissues were stored at -80°C or in ethanol at room temperature.

2.2. DNA Extraction

DNA extraction was performed using a QIAamp DNA Mini kit (QIAGEN, Australia) following the 'Tissue' protocol with final elution into 100 µL. Each neat DNA extract (5 µL) was run on a 2% agarose gel stained with ethidium bromide to visualise quality and quantity of the extracted DNA. DNA extracts were also quantified using a Qubit fluorometer (Life Technologies, NSW, Australia) and diluted to 1 ng/µL to ensure optimal and consistent sample DNA concentration for multiplexing.

2.3. Identification of novel STR loci

Identification of new STR markers for application to multiplexing was conducted following workflows suggested for identifying new STR markers using Mass Parallel Sequencing [35,36].

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A DNA extract was purified and sent to the Australian Genome Research Facility (AGRF) (Qld., Australia) for Mass Parallel Sequencing using 1/16 plate on a 454 Next Generation Sequencer (Roche). Mass Parallel Sequence data were analysed using QDD [37]. Sequences were sorted by sequence tag. Duplicate sequences in the dataset were removed or contigs created where sequence identity was >95%. Parameters were set to return sequences with a minimum of 80 base pairs (bp) containing simple and compound microsatellite motifs of 2-6 bp repeats and ≥ 5 repeat units were selected. Primer3 [38] was used to design primers to amplify putative STRs with a PCR product size range of 350-550 bp. Multiplex Ready Technology [39] was used to incorporate fluorescent dyes into PCR products for visualisation by 3130xl capillary electrophoresis. Similarly to M13 tagging [40], this enabled testing of the polymorphic nature of the putative loci without expensive fluorescent labelling of every primer pair. Locus specific primer pairs containing an MRT tag sequence and associated tag sequence primers (forward fluorescently labelled) were synthesised by Integrated DNA Technologies (Iowa, USA). Amplification method followed that of Ciavaglia *et al.* [41]. Each locus was amplified in singleplex using six python DNA extracts from diverse localities across the species range to survey for initial locus polymorphism and heterozygosity.

2.4. Multiplex Assay Design and Optimisation

Three different multiplex assays were constructed following guidance of Butler [42], designated Light, Medium and Dark, and initially incorporating 27 loci. Primers designated "MS", developed by Jordan *et al.* [30], were included for cross-compatibility with previous studies. The novel loci developed during this study have the prefix "MsF". Three loci were included in all of the multiplex assays as quality assurance markers. This three locus redundancy allowed reproducibility of allele calls to be assessed between multiplex assays and can flag potential sample mix up between assays. Primers were examined by eye and run through http://www.thermoscientificbio.com/webtools/multipleprimer/ to test for self-complementarity and complementarity between primers within the multiplex. A single guanine base or GTTTCTT 'PIG-tail' was added to the 5' end of every reverse primer to facilitate complete adenylation of PCR products and accurate single base precision genotyping [43]. Locus-specific primer pairs were ordered for the final three assay primer set (fluorescently labelled primers from Applied Biosystems (CA, USA) and unlabelled primers from Integrated DNA Technologies (IA, USA) and Geneworks (Adelaide, Australia)).

Multiplex assays were optimised in a 25 µL reaction volume using 12.5 µL Multiplex PCR Mastermix (Qiagen, Australia), 11.5 µL primer mix (primers present in varying concentrations) and 1 ng template DNA. PCR conditions were: initial activation at 95°C for 15 minutes, followed by 29 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s, and extension at 72°C for 60 s, and concluded with a final extension step of 60°C for 30 minutes to promote complete product adenylation [44–46].

Multiplex PCR products were visualised by capillary electrophoresis using a 3130xl (Applied Biosystems) with a 36 cm capillary array and POP-4 polymer. Spectral calibration was initially conducted using the G5 dye set matrix standard (Applied Biosystems). PCR product (1 µL) was added to 9.7 µL Hi-Di Formamide and 0.3 µL Liz600 internal size standard (Life Technologies, USA). At least one allelic ladder was included per 31 samples, three allelic ladders were included on a 96 well plate to account for any potential migration shift across the six injections. Data were analysed

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using GeneMapper ID v3.0 with panel and bin sets developed for each multiplex assay (available from the authors on request). Samples displaying spectral saturation, resulting in pull-up peaks in neighbouring dye sets, were rerun at a dilution of either 1/50 or 1/100 based on the degree of pull up (necessary only very rarely due to quantified starting template).

2.5. Construction of an allelic ladder

An initial screening dataset (n=43) was examined to identify all unique alleles at each locus. Heterozygous individuals were chosen that represented the majority of alleles present without duplication of an allele between two individuals. Singleplex PCRs were performed in 25 µL reactions using 2x My Taq Hotstart Mix (Bioline, Australia), 0.4 µM each primer and 0.5-3 µL additions of DNA template for each of the heterozygotes chosen for the specific locus, resulting in a DNA mixture of alleles per locus. A second round of singleplex reactions were performed including only DNA template from individuals exhibiting low peaks and first and second round PCR products pooled to achieve greater balance in each single locus DNA mixture. Singleplex mixtures for each multiplex assay were pooled and visualised by fragment analysis. More singleplex product was added to strengthen poorly amplifying loci in the resulting ladder profile. Subsequent alleles were added to ladders by addition of mixed DNA singleplex template to the pooled ladder sample to improve allelic representation in the ladders as more alleles became known. Allelic ladder replication was trialled for all three multiplex ladders via re-amplification of each respective ladder template diluted to both 1/1000 and 1/1000000 , following cycling conditions described in 2.4 with cycle number varied to 15, 22 and 29 total amplification cycles (18 amplifications in total).

2.6. Sample genotyping

The three multiplex primer assays were used to genotype native carpet pythons collected extensively from across the entire geographic range of the species. Samples were genotyped using allele calls corresponding to the underlying base motif repeat number (in accordance with recommendation 7 of [32]).

2.7. Validation

2.7.1. Reproducibility

During multiplex assay optimisation, one sample was included on every run for comparative purposes. Multiple optimisation assays were runs on the 3130xl instrument at Flinders University. One PCR plate incorporating all three multiplex assays was run on both a 3130xl instrument at Forensic Science SA and the Flinders University 3130xl instrument. Repeatability and reproducibility data were collated from six, seven and eight runs for the Light, Medium and Dark assays, respectively.

2.7.2. Heterozygote balance

Profile data were collated from samples genotyped using the three optimised multiplex assays and validation tests. Only loci exhibiting heterozygote peaks >1000 RFU were included, as average peak height has a recognised inverse correlation with variability in heterozygote balance (H_b)[47]. This

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also avoided samples for which poor DNA quality led to a 'ski slope' effect of imbalance in alleles of large size differences. The heterozygote balance of each allele pair was calculated by:

$H_b = \phi HMW/\phi LMW$

where ϕHMW is the height in RFU of the high molecular weight product and ϕLMW is the height of the low molecular weight product. The mean, variance and standard deviation of H_b were calculated per locus.

2.7.3. Species specificity

Cross-species amplifications were performed on a range of snake species described previously [41], including *Antaresia childreni*, *A. stimsoni*, *A. maculosa*, *Aspidites melanocephalus*, *A. ramsayi*, *Liasis olivaceus*, *L. mackloti*, *Python reticulatus*, *P.timoriensis*, *P. curtus*, *P. brongersmai* and *P. breitensteini*. Human DNA (1 ng) was tested for cross-reactivity.

2.7.4. Assay Sensitivity

The three multiplex assays were amplified using DNA template diluted to decreasing concentrations to ascertain the lower limit for DNA profiling. Amplifications were performed as per section 2.4 with addition of the following amounts of DNA template: 5 ng, 2 ng, 1 ng, 500 pg, 250 pg, 125 pg, 62.5 pg, 31.25 pg, 15.63 pg, 7.8 pg, 4 pg and 2 pg.

2.7.5. Repeat motif sequence determination by Sanger sequencing

Homozygote individuals were amplified in singleplex reactions using unlabelled primers. Amplification reactions contained 0.5-1 µL extracted DNA template (~1-5 ng), 200 nM dNTPs, 1.5 mM $MgCl_2$, 1.25 µM of each primer and 1 unit of Mango Taq DNA Polymerase (BIOLINE, Australia) in a total volume of 20 µL. Cycling conditions were 35 cycles of: denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 15 s. Successful product amplification was confirmed and DNA concentration estimated by 2% agarose gel electrophoresis and visualization by ethidium bromide (10 mg/mL) using a Bio-Rad Gel Doc and Image Lab Software (Bio-Rad, Australia). PCR products were cleaned up in preparation for sequencing using ExoSap (GE Healthcare, Australia), following the manufacturer's protocol. DNA sequencing using either forward or reverse primer was performed by the AGRF (Adelaide, Australia). Sequence alignment and editing was performed using Geneious v5.6.7 (Biomatters).

2.8. Reference database construction and quality control

Genotypes obtained from the three multiplex assays were collated in Excel (Microsoft). Replicate genotyping of a single individual was examined for concurrent results. Duplicate genotypes were removed. Any samples not *Morelia spilota* or *M. bredli,* with unspecified locality data, identified as captive or hybrid, or exhibiting mixed profiles or profile ambiguities, were removed from the dataset. Homozygote loci that exhibited inter-locus peak balance patterns suggestive of the presence of a null heterozygote allele caused by a primer binding site mutation were excluded. Allele identities still in doubt were specified as unknown and genotypes were removed from the dataset if any suspicion remained about their identity. Loci exhibiting problematic genotyping were excluded from the database.

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Allele calls were converted directly to absolute base pair designations using an Excel Macro function for ease of downstream computational analysis. Three alleles displayed unusual migrations patterns, repeatedly migrating a fraction of a base pair different in size from all other alleles called by the same bin. This is likely due to sequence polymorphism in the underlying complex repeat motifs of the four loci. As the largest allele identified was 525 bp, the four alleles designated were allocated unique absolute base pair designations 600-602 to differentiate as distinct alleles for subsequent analyses.

2.9. STR database characteristics

GenAlEx v6.5 [48,49] was used to calculate population genetic parameters and estimate discrimination power. Estimates of the least and most common genotypes present in the population were calculated [50]. The rarest allele frequency was calculated using the 5/2N minimum allele frequency rule [44,51].

3. Results and Discussion

3.1. Identification of Novel STR Loci

A total of 133,193 raw sequence reads were returned from Mass Parallel Sequencing, yielding 7486 sequences containing unique STR loci. Primers were designed for 3225 loci. Sixty-five unique simple tetra- and penta-nucleotide repeat motifs were shortlisted. The maximum size of sequences containing simple motifs was 267 base pairs so loci with compound and complex motifs as well as sequences constructed of contigs were included to increase choices of amplicons of the high molecular weight range for multiplexing. A total of 167 putative STR loci were available for consideration with the longest amplification fragment size being 543 bp. Thirty-six primer pairs were ordered with MRT tag sequences incorporated. Locus characteristics have been reported previously [52]. Initial screening of fourteen individuals yielded seven loci that were discarded due to >2 amplification products, absence of amplification products or very poor peak morphology. Of the remaining loci, allelic diversity ranged between 5-16 alleles. Locus size (distance between smallest and largest allele) ranged from 16-81 bp.

3.2. Multiplex optimisation and allelic ladders

Primer redesign and multiplex rearrangement was required following initial screening of 49 samples to rectify overlaps that became apparent in allele size ranges at respective loci. Two poorly performing loci were removed and the quality markers redesignated. The final multiplex marker set comprises three 11-plex profiling assays, incorporating 27 loci. Table 1 provides details of the three final optimised multiplexes including STR locus name, motif type, primer sequences, fluorophore label and optimised primer concentrations to enable effective multiplex screening. One individual's complete DNA profile using all three multiplex assays is provided (Figure 1) along with an example of one of the three allelic ladders (Figure 2). While inclusion of all alleles seen during screening in the allelic ladders was not possible, the allelic ladders collectively contain a range of 4 to 16 alleles for all 27 loci. The optimum allelic ladder reproduction resulted from a 1/1000 dilution amplified for fifteen cycles.

3.3. Sample genotyping

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A total of 304 carpet pythons were profiled for 27 loci. Of the 27 loci initially genotyped, three loci MS2, MS16 and MsF14 were removed during quality assurance evaluation. This was due to genotyping difficulties caused by poor morphology (see Figure 3 for MS2 example) reflecting complex underlying repeat motifs at two loci and an STR present in a primer sequence causing population specific incomplete amplification or allelic drop out at MsF14 [7] (this marker is potentially very interesting for population differentiation and warrants ongoing research into its potential application to casework). The allelic ladder is comprehensive for these 27 loci, where 24 currently provide accurate genotype designation. Three loci require further work to determine the limitations of their work and currently under-perform. We suggest omission of these three loci for casework as interpretation of results is complicated and unnecessary with 24 highly discriminating loci.

Complete 24 locus profiles were obtained from 190 individuals, while 52 individuals exhibited one incomplete locus and 12 were missing two loci. Only 9 individuals exhibited no amplification product and 36 individuals exhibited alleles at 19 loci or less.

3.4. Repeat motif sequence determination by Sanger sequencing

All sequences containing complete STR regions have been deposited in Genbank (Accession Numbers pending).

A total of 141 sequences were attained. Of the 27 original loci included in this study, only MS16 did not give meaningful sequence that revealed the nature of the underlying repeat motif. Sequences obtained from loci with complex underlying repeat motifs were very difficult to align due to the vast differences in the underlying sequence content (figure 3).

3.5. Validation testing

3.5.1. Reproducibility

Of the remaining 24 loci, 19 complete loci (34 alleles) exhibited less than 1.00 absolute base pair migration variability between all runs over all assays, obtained using different instruments in two independent laboratories. An average migration range of 0.61 bp was obtained over the 34 alleles. The homozygote allele at quality marker MsF31 exhibited 0.81 bp migration variability over 21 runs. Five loci exhibited alleles with >1.00 absolute base pair variability across runs, which could confound allele calling with confidence to single base pair accuracy. The results emphasise the importance of including an allelic ladder with every run - already widely recognised as an important component of any wildlife forensic genotyping assay [51].

3.5.2. Heterozygote balance

Figure 4 provides the mean heterozygote balance seen per locus. The error bars represent 95% confidence intervals. Dashed lines represent 60% balance in either direction (ϕHMW peak larger than ϕLMW peak or vice versa), included to indicate the traditional recommended threshold of balance at a single source locus [53], prior to the development of continuous models. Only three loci exceed the balance threshold (2σ), but all exhibit H_b greater than 50%. All but one locus exhibit a mean balance favouring increased amplification of the LMW product, demonstrating the recognised amplification efficiency bias towards smaller DNA templates and therefore alleles containing less repeat units [54].

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3.5.3. Species specificity

Results of the species specificity study are reported previously in Ciavaglia *et al.* [41]. Human DNA failed to amplify any product; this is important to ensure that contaminating human DNA transfer at the time of collection or previous handling will not compromise the result.

3.5.4. Assay sensitivity

All 24 loci were successfully amplified using a DNA template at 0.5 ng and above. Spectral saturation or pull-up was observed when using template concentration of 5 ng and 2 ng (Figure 5). Optimal results were obtained using 1 ng (Figure 1) with no pull-up observed and strong amplification of all alleles. At 250 pg all loci showed amplification products in the light and dark assays, but only one locus (MS4h) was lost from the medium assay. Addition of 125 pg template results in homozygote peaks are below 150 relative fluorescent units (RFU) demonstrating potential for allelic dropout at this template. Figure 5 shows attrition of complete loci in the light assay beginning around 62.5 pg. Partial amplification was seen down to 31.25 pg template and below this amount no genuine locus amplification was detected in the light and medium assays. Five to six alleles were designated at 15 pg/µL DNA input in the dark assay, but below this in-put mass any potential allele peaks could not be confidently called as such. Determination of thresholds for allelic dropout of loci included in these assays is beyond the scope of this study, but should be conducted empirically by any laboratory planning to implement these profiling assays in casework.

3.6. Reference database construction and quality control

A 24 locus reference genotype database was constructed including 249 individuals (Supplementary Material 1 specifies individuals included in the reference database). The choice of data included in the final reference dataset is a compromise between maximising the database size and minimising the amount of missing data present in partial profiles. A threshold of 22+ complete loci was chosen as the most appropriate final reference dataset. The database includes 52 and 12 individuals with incomplete data at one or two loci respectively. The resulting allele frequency database is presented (Table 2).

3.7. STR database characteristics

A total of 752 alleles were observed across 24 loci screened in 249 individuals. Allelic diversity was very high across all loci genotyped, ranging between 10 alleles and 88 alleles per locus (Table 2). Observed heterozygosity ranged between 0.397 and 0.865. Mean observed heterozygosity was 0.671, indicating the high potential of the marker set to be powerful markers for discrimination between individuals. The most common and rarest genotype estimates were 1 in 10^{29} and 1 in 10^{48}. The probability of obtaining an identical genotype from two individuals chosen at random from the population, assuming that the individuals are siblings (Probability of Identity of Siblings, PI_{SIBS}) was 1 in 10^{-12}.

The loci presented deviate significantly from Hardy-Weinberg equilibrium. Preliminary population genetic analysis (unpublished data) has confirmed that this is due to population substructure within this geographically wide ranging dataset. Subpopulations have demonstrated intra-locus allelic independence, indicating that none of the markers are sex-linked. The PI_{SIBS} is calculated because the degree of population substructure present within this dataset is yet to be established. The kinship

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factor of 0.5 applied to a sibling relationship is a much more conservative figure than most co-ancestry coefficient corrections for substructure seen in wild populations. This calculation is expected to give a more conservative figure than a calculation involving Θ based on empirically measured population substructure.

3.8. Application to casework

The authors wish to stress here that the above statistics are presented to illustrate the strong discriminatory potential of these STR markers in carpet pythons. Work is ongoing to establish the degree of population genetic structuring present in this dataset and its capability to provide statistical support for the types of questions arising in forensic investigations of crimes involving carpet pythons. The current reference dataset provides an overview of the genetic variability of snakes across Australia, but further population genetic analysis is required to determine the degree of population substructure present that will influence the statistical conclusions presented in any forensic analyses.

We present a number of tests common to developmental validation for forensic application. It is however, recommended that further validation studies are conducted examining sample specificity, marker inheritance and mutation rates, environmental degradation, and ideally an inter-laboratory collaborative study performed using positive control DNA and blind trial samples to confirm the robustness of the marker set and achieve full validation. The allelic ladders will assist with inter-laboratory comparisons of known positive control DNA samples and both of these components are available from the authors upon request. Verification (sometimes referred to as internal validation) needs to be conducted prior to casework application to determine the abilities and limitations of the assays using instrumentation specific to the investigating laboratory. Determination of locus dependent stutter ratios and mixture studies might also have utility if evidentiary items involving mixed DNA templates are anticipated in casework.

4. Conclusion

We have constructed three STR multiplex assays, each with 11 hypervariable loci, for the effective assignment of an unknown sample to a reference sample or to a mini-DNA database. Quality control loci are included in all three multiplex assays, allowing concordant genotyping between the three tests to ensure reproducibility and minimize the opportunity for error. The number of identified alleles for each of the 24 loci varied from 10 to 88 leading to an extremely large number of possible genotypes. Three comprehensive allelic ladders have been created enabling accurate genotyping. These ladders will also allow inter-laboratory comparisons to be made. A number of studies common to developmental validation are presented herein, but additional validation tests are suggested and verification is required prior to implementation for casework. The power of association between two matching DNA profiles is extremely high, such that a conservative kinship factor will still lead to high powers of association. The work presented here is not only a means to identify questioned samples as being from a particular python, but also a model for other such STR multiplex assays for wildlife species implicated in crimes.

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7. Conflicts of interest

None

References

[1] UNODC, World Wildlife Crime Report: Trafficking in protected species, New York, 2016. http://www.unodc.org/unodc/en/data-and-analysis/wildlife.html.

[2] NIST, OSAC Organizational Structure, (2017). https://www.nist.gov/topics/forensic-science/osac-organizational-structure (accessed October 6, 2017).

[3] E. Alacs, A. Georges, Wildlife across our borders: a review of the illegal trade in Australia, Aust. J. Forensic Sci. 40 (2008) 147–160.

[4] J. Herbig, The illegal reptile trade as a form of conservation crime: a South African criminological investigation, in: R. White (Ed.), Glob. Environ. Harm Criminol. Perspect., Taylor & Francis, 2013: pp. 110–131. https://books.google.co.uk/books?hl=en&lr=&id=N2gQBAAAQBAJ&oi=fnd&pg=PA110&dq=reptiles+are+easily+concealed+in+trade&ots=RZaDD9yVHh&sig=-s5HbDO-emwins64Vd4MXtStEq0#v=onepage&q=reptiles are easily concealed in trade&f=false.

[5] G.E. Rosen, K.F. Smith, Summarizing the evidence on the international trade in illegal wildlife, Ecohealth. 7 (2010) 24–32. doi:10.1007/s10393-010-0317-y.

[6] C. Barry, Rogue Traders, Aust. Geogr. Mag. (2011) 124–130.

[7] S. Ciavaglia, H. Dridan, K.P. Kirkbride, A. Linacre, Current issues with the investigation of wildlife crime in Australia: Problems and opportunities for improvement, J. Int. Wildl. Law Policy. 18 (2015) 244–263. doi:10.1080/13880292.2015.1074008.

ACCEPTED MANUSCRIPT

[8] M. Poetsch, S. Seefeldt, M. Maschke, E. Lignitz, Analysis of microsatellite polymorphism in red deer, roe deer, and fallow deer–possible employment in forensic applications, Forensic Sci. Int. 116 (2001) 1–8. http://www.sciencedirect.com/science?_ob=MImg&_imagekey=B6T6W-41WJBSP-1-B&_cdi=5041&_user=131115&_pii=S0379073800003376&_orig=search&_coverDate=02/01/2001&_sk=998839998&view=c&wchp=dGLbVlz-zSkWb&md5=2d9b08f438ffb9c671c879de48ab77c1&ie=/sdarticle.pdf.

[9] R.M. Jobin, D. Patterson, Y. Zhang, DNA typing in populations of mule deer for forensic use in the Province of Alberta, Forensic Sci. Int. Genet. 2 (2008) 190–197. doi:10.1016/j.fsigen.2008.01.003.

[10] E. Socratous, E. a. M. Graham, G.N. Rutty, Forensic DNA profiling of Cervus elaphus species in the United Kingdom, Forensic Sci. Int. Genet. Suppl. Ser. 2 (2009) 281–282. doi:10.1016/j.fsigss.2009.08.127.

[11] S. Caratti, L. Rossi, B. Sona, S. Origlia, S. Viara, G. Martano, C. Torre, C. Robino, Analysis of 11 tetrameric STRs in wild boars for forensic purposes, Forensic Sci. Int. Genet. 4 (2010) 339–342. doi:10.1016/j.fsigen.2010.07.001.

[12] C.R. Primmer, M.T. Koskinen, J. Piironen, The one that did not get away: individual assignment using microsatellite data detects a case of fishing competition fraud., Proc. Biol. Sci. 267 (2000) 1699–1704. doi:10.1098/rspb.2000.1197.

[13] J.P. Grobler, A. Kotze, H. Swart, E.M. Hallerman, The application of microsatellite DNA markers for forensic analysis of koi carp (Cyprinus carpio), S. Afr. J. Sci. 101 (2005) 19–21.

[14] H.G. Eiken, R.J. Andreassen, A. Kopatz, S.G. Bjervamoen, I. Wartiainen, C. Tobiassen, P.M. Knappskog, P.E. Aspholm, M.E. Smith, J. Aspi, Population data for 12 STR loci in Northern European brown bear (Ursus arctos) and application of DNA profiles for forensic casework, Forensic Sci. Int. Genet. Suppl. Ser. 2 (2009) 273–274. doi:10.1016/j.fsigss.2009.07.007.

[15] S. Mondol, N. R, V. Athreya, K. Sunagar, V. Selvaraj, U. Ramakrishnan, A panel of microsatellites to individually identify leopards and its application to leopard monitoring in human dominated landscapes, BMC Genet. 10 (2009) 79. doi:10.1186/1471-2156-10-79.

[16] R. Caniglia, E. Fabbri, C. Greco, M. Galaverni, E. Randi, Forensic DNA against wildlife poaching: Identification of a serial wolf killing in Italy, Forensic Sci. Int. Genet. 4 (2010) 334–338. doi:10.1016/j.fsigen.2009.10.012.

[17] N. Dawnay, R. Ogden, R.S. Thorpe, L.C. Pope, D.A. Dawson, R. McEwing, A forensic STR profiling system for the Eurasian badger: A framework for developing profiling systems for wildlife species, Forensic Sci. Int. Genet. 2 (2008) 47–53. doi:10.1016/j.fsigen.2007.08.006.

[18] R. Ogden, N. Dawnay, R. McEwing, Development of STR profiling systems for individual identification in wildlife: A case study of the Eurasian badger, Meles meles, Forensic Sci. Int. Genet. Suppl. Ser. 1 (2008) 612–613. doi:10.1016/j.fsigss.2007.10.224.

[19] M. Wesselink, I. Kuiper, Individual identification of fox (Vulpes vulpes) in forensic wildlife investigations, Forensic Sci. Int. Genet. Suppl. Ser. 3 (2011) e214–e215. doi:10.1016/j.fsigss.2011.08.107.

[20] B. Van Asch, C. Alves, F. Pereira, L. Gusmão, A. Amorim, A new autosomal STR multiplex for canine genotyping, Forensic Sci. Int. Genet. Suppl. Ser. 1 (2008) 628–629. doi:10.1016/j.fsigss.2007.10.043.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

[21] R. Ogden, R.J. Mellanby, D. Clements, A.G. Gow, R. Powell, R. McEwing, Genetic data from 15 STR loci for forensic individual identification and parentage analyses in UK domestic dogs (Canis lupus familiaris), Forensic Sci. Int. Genet. 6 (2012) e63–e65. doi:10.1016/j.fsigen.2011.04.015.

[22] E. Wictum, T. Kun, C. Lindquist, J. Malvick, D. Vankan, B. Sacks, Developmental validation of DogFiler, a novel multiplex for canine DNA profiling in forensic casework, Forensic Sci. Int. Genet. 7 (2013) 82–91. doi:10.1016/j.fsigen.2012.07.001.

[23] N. Dawnay, R. Ogden, J.H. Wetton, R.S. Thorpe, R. McEwing, Genetic data from 28 STR loci for forensic individual identification and parentage analyses in 6 bird of prey species, Forensic Sci. Int. Genet. 3 (2009). doi:10.1016/j.fsigen.2008.07.001.

[24] N.E. White, R. Dawson, M.L. Coghlan, S.R. Tridico, P.R. Mawson, J. Haile, M. Bunce, Application of STR markers in wildlife forensic casework involving Australian black-cockatoos (Calyptorhynchus spp.), Forensic Sci. Int. Genet. 6 (2012) 664–670. doi:10.1016/j.fsigen.2011.10.003.

[25] B. Berger, C. Berger, W. Hecht, A. Hellmann, U. Rohleder, U. Schleenbecker, W. Parson, Validation of two canine STR multiplex-assays following the ISFG recommendations for non-human DNA analysis, Forensic Sci. Int. Genet. 8 (2014) 90–100. http://dx.doi.org/10.1016/j.fsigen.2013.07.002.

[26] M.J.C. Van Hoppe, M.A.V. Dy, M. Van Den Einden, A. Iyengar, SkydancerPlex: A novel STR multiplex validated for forensic use in the hen harrier (Circus cyaneus), Forensic Sci. Int. Genet. 22 (2016) 100–109. doi:10.1016/j.fsigen.2016.02.003.

[27] S.K. Wasser, C. Mailand, R. Booth, B. Mutayoba, E. Kisamo, B. Clark, M. Stephens, Using DNA to track the origin of the largest ivory seizure since the 1989 trade ban., Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 4228–4233. doi:10.1073/pnas.0609714104.

[28] S.K. Wasser, W. Joseph Clark, O. Drori, E. Stephen Kisamo, C. Mailand, B. Mutayoba, M. Stephens, Combating the illegal trade in African elephant ivory with DNA forensics, Conserv. Biol. 22 (2008) 1065–1071. doi:10.1111/j.1523-1739.2008.01012.x.

[29] S.K. Wasser, A.M. Shedlock, K. Comstock, E. a Ostrander, B. Mutayoba, M. Stephens, Assigning African elephant DNA to geographic region of origin: applications to the ivory trade., Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 14847–14852. doi:10.1073/pnas.0403170101.

[30] P.W. Jordan, a E. Goodman, S. Donnellan, Microsatellite primers for Australian and New Guinean pythons isolated with an efficient marker development method for related species, Mol. Ecol. Notes. 2 (2002) 78–82. doi:10.1046/j.1471-8278 .2001.00135.x.

[31] D. Taylor, Using DNA markers for wildlife management and protection: a study of the population structure and systematics of the Australian carpet pythons (Reptilia: Morelia spilota complex), Flinders University, SA, 2005.

[32] A. Linacre, L. Gusmao, W. Hecht, A.P. Hellmann, W.R. Mayr, W. Parson, M. Prinz, P.M. Schneider, N. Morling, ISFG: Recommendations regarding the use of non-human (animal) DNA in forensic genetic investigations, Forensic Sci. Int. Genet. 5 (2011) 501–505. doi:10.1016/j.fsigen.2010.10.017.

[33] S.W.G. on D.A.M. (SWGDAM), Validation Guidelines for DNA Analysis Methods, (2012). https://docs.wixstatic.com/ugd/4344b0_813b241e8944497e99b9c45b163b76bd.pdf (accessed October 6, 2017).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

[34] S. Ciavaglia, S. Donnellan, J. Henry, A. Linacre, Species identification of protected carpet pythons suitable for degraded forensic samples, Forensic Sci. Med. Pathol. 10 (2014) 295–305. doi:10.1007/s12024-014-9573-4.

[35] M.G. Gardner, A.J. Fitch, T. Bertozzi, A.J. Lowe, Rise of the machines - recommendations for ecologists when using next generation sequencing for microsatellite development, Mol. Ecol. Resour. 11 (2011) 1093–1101. doi:10.1111/j.1755-0998.2011.03037.x.

[36] E. Guichoux, L. Lagache, S. Wagner, P. Chaumeil, P. L??ger, O. Lepais, C. Lepoittevin, T. Malausa, E. Revardel, F. Salin, R.J. Petit, Current trends in microsatellite genotyping, Mol. Ecol. Resour. 11 (2011) 591–611. doi:10.1111/j.1755-0998.2011.03014.x.

[37] E. Meglécz, C. Costedoat, V. Dubut, A. Gilles, T. Malausa, N. Pech, J.F. Martin, QDD: A user-friendly program to select microsatellite markers and design primers from large sequencing projects, Bioinformatics. 26 (2010) 403–404. doi:10.1093/bioinformatics/btp670.

[38] A. Untergasser, I. Cutcutache, T. Koressaar, J. Ye, B.C. Faircloth, M. Remm, S.G. Rozen, Primer3-new capabilities and interfaces, Nucleic Acids Res. 40 (2012) 1–12. doi:10.1093/nar/gks596.

[39] M.J. Hayden, T.M. Nguyen, A. Waterman, K.J. Chalmers, Multiplex-ready PCR: a new method for multiplexed SSR and SNP genotyping., BMC Genomics. 9 (2008) 80. doi:10.1186/1471-2164-9-80.

[40] M. Schuelke, An economic method for the fluorescent labeling of PCR fragments, Nat. Biotechnol. 18 (2000) 233–234. doi:10.1038/72708.

[41] S. Ciavaglia, H. Dridan, A. Linacre, Getting more for less : can forensic tools for Australian wildlife enforcement support international compliance efforts ?, Aust. J. Forensic Sci. 618 (2017) 1–10. doi:10.1080/00450618.2017.1384060.

[42] J.M. Butler, Constructing STR multiplex assays., in: A. Carracedo (Ed.), Forensic DNA Typing Protoc., Springer Science & Business Media, 2005: pp. 53–66. doi:10.1111/j.1556-4029.2008.00595.x.

[43] M.J. Brownstein, J.D. Carpten, J.R. Smith, Modulation of non-templated nucleotide addition by Taq DNA polymerase: Primer modifications that facilitate genotyping, Biotechniques. 20 (1996) 1004–1010.

[44] J.M. Butler, Forensic DNA Typing: biology, technology and genetics of STR markers, Elsevier Academic Press, 2005.

[45] C.P. Kimpton, P. Gill, A. Walton, A. Urquhart, E.S. Millican, M. Adams, Automated DNA profiling employing multiplex amplification of short tandem repeat loci., Genome Res. 3 (1993) 13–22.

[46] Applied Biosystems, AmpF l STR ® SGM Plus ® PCR Amplification Kit user guide, (2012) 137.

[47] J.A. Bright, D. Taylor, J.M. Curran, J.S. Buckleton, Developing allelic and stutter peak height models for a continuous method of DNA interpretation, Forensic Sci. Int. Genet. 7 (2013) 296–304. doi:10.1016/j.fsigen.2012.11.013.

[48] R. Peakall, P.E. Smouse, GenALEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update, Bioinformatics. 28 (2012) 2537–2539. doi:10.1093/bioinformatics/bts460.

ACCEPTED MANUSCRIPT

[49] R. Peakall, P.E. Smouse, GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research, Mol. Ecol. Notes. 6 (2006) 288–295. doi:10.1111/j.1471-8286.2005.01155.x.

[50] J.M. Butler, Advanced topics in forensic DNA typing: interpretation, Academic Press, 2014.

[51] R.N. Johnson, L. Wilson-Wilde, A. Linacre, Current and future directions of DNA in wildlife forensic science, Forensic Sci. Int. Genet. 10 (2014) 1–11. doi:10.1016/j.fsigen.2013.12.007.

[52] S.A. Ciavaglia, J.M. Henry, A.M.T. Linacre, Profiling pythons to combat common illegal wildlife activities, Forensic Sci. Int. Genet. Suppl. Ser. 4 (2013) e31–e32. doi:10.1016/j.fsigss.2013.10.015.

[53] P. Gill, C.H. Brenner, J.S. Buckleton, A. Carracedo, M. Krawczak, W.R. Mayr, N. Morling, M. Prinz, P.M. Schneider, B.S. Weir, DNA commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures, Forensic Sci. Int. 160 (2006) 90–101. doi:10.1016/j.forsciint.2006.04.009.

[54] J.S. Buckleton, J.-A. Bright, D. Taylor, eds., Forensic DNA Evidence Interpretation, Second ed., CRC Press, Taylor & Francis Group, 2016.

Figure and Table Captions

Figure 1: A complete STR profile is shown for one individual comprising the dark, medium and light multiplex assays. Vertical shades per locus denote allelic ladder bins. Concordant genotypes can be seen across the three quality markers, MsF5 (FAM dye), MsF31 (VIC dye) and MsF22 (PET dye). Peak labels include (descending order): allele call, absolute base size, relative fluorescent units (RFU).

Figure 2: Allelic ladder developed for the dark multiplex assay. The horizontal shading in each locus are bins used by the genotyping software to designate allele number to a peak. Each bin represents a known allele at the locus. Grey bins represent complete motifs and red bins represent micro-variant alleles.

Figure 3: The complex underlying repeat motif at locus MS2, involving both di-nucleotide and tetra-nucleotide repeat motifs that exhibited non-uniform patterns across individuals, complicated sequence alignment and caused increased stutter artefacts (characteristic of di-nucleotide repeats). Poor peak morphology was often seen in alleles of MS2 (inset), complicating genotyping to the extent that confident calling to single base pair resolution was not possible.

Figure 4: Heterozygote balance (H_b) per locus. Sample size per locus is provided in brackets. Points represent mean H_b and error bars represent 95% confidence intervals. H_b is provided as a ratio to differentiate cases where either the HMW or LMW peak exhibits the greater peak height. Dashed lines represent the 60% balance threshold recommended for single source profiles.

Figure 5: Examples of the effect of non-optimal DNA template amounts on profile quality obtained during the sensitivity study: a) increased template amount (2 ng) results in a strong profile with pull-up peaks from locus MsF31 evident in FAM and PET dyes, and b) decreased starting template (62.5 pg) gives a weak profile exhibiting attrition of one locus (MsF33), heterozygote imbalance and increased possibility of allelic dropout.

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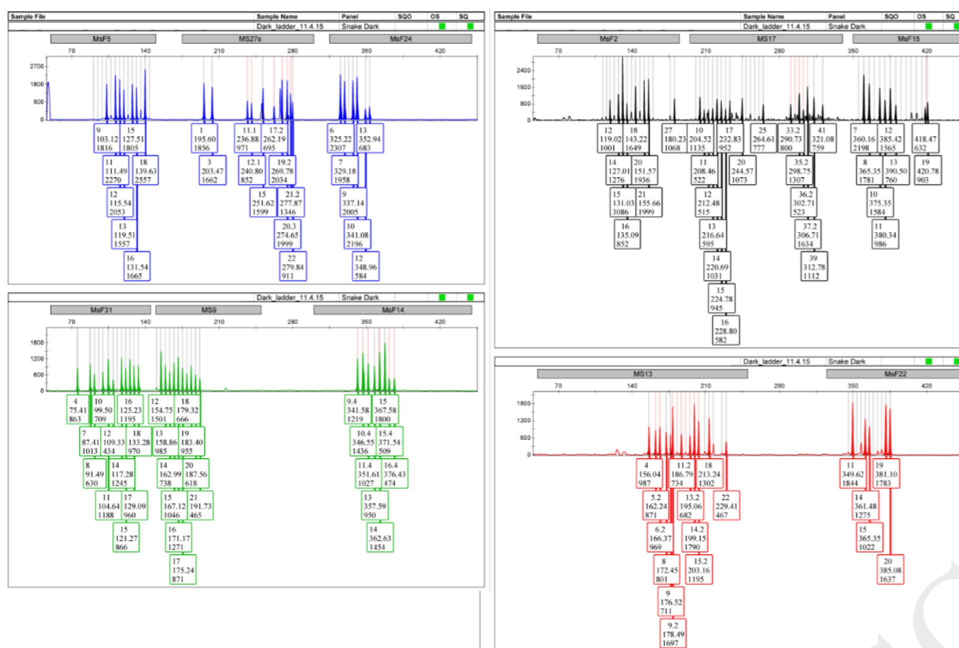

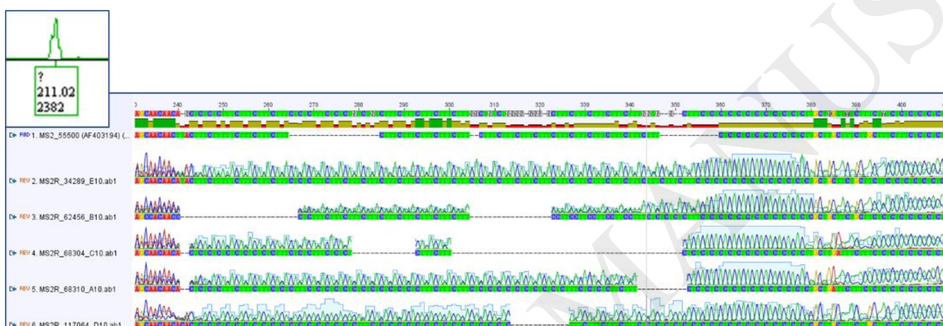

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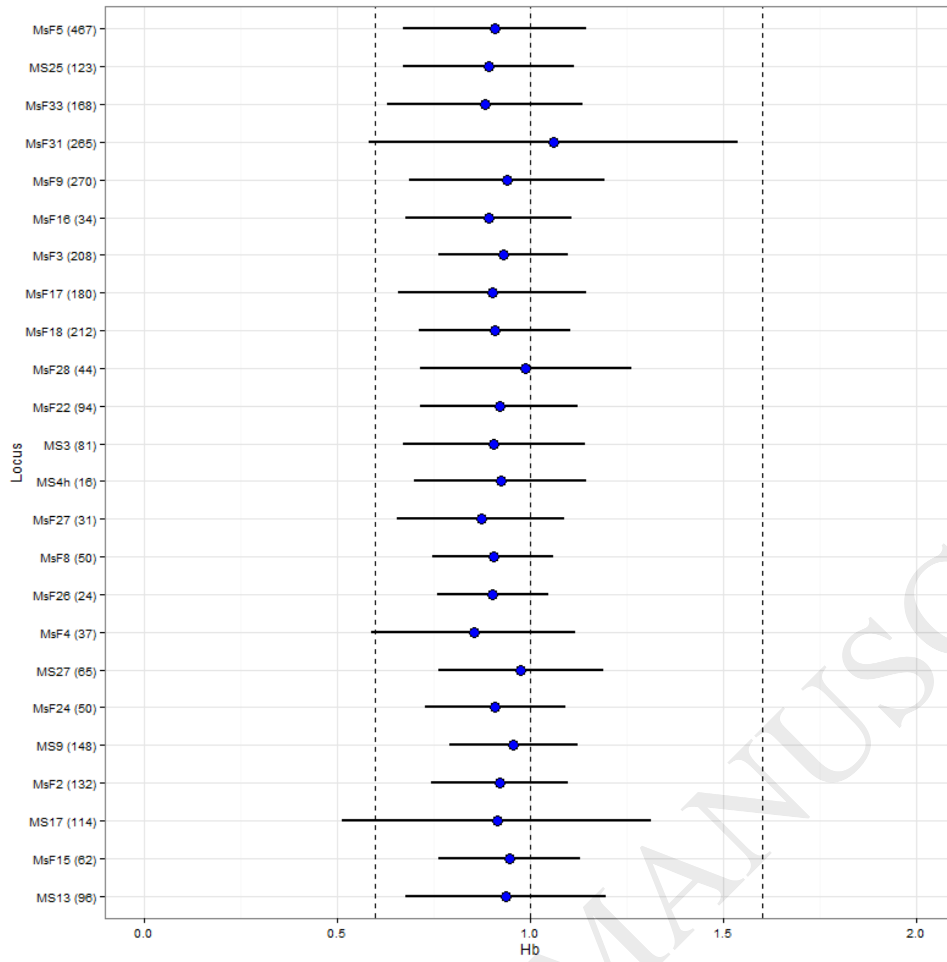

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a)

b)

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Table 1: Details of three optimised multiplex assays, including optimised primer concentrations that yield a balanced multiplex profile. "MsF" loci have been developed during this study and "MS" primers loci included for cross-compatibility with previous work. Primer suffixes denote primers redesigned for multiplex purposes: h – higher T_m; s – smaller product size; t – tag added for complete adenylation. Locus MS4h, MS3t and MS27s names include redesigned primer designations to avoid confusion between these products and products reported in previous studies.

Assay	Locus	Dye	Motif	Primer name	Primer sequence (5'-3')	Locus range (bp)	Primer concentration
Light							
	MsF5	6-FAM	$(TAGA)_n$	MsF5F_FAM	AGCTGCCCAAAGTTGCTATG	50-150	60 nM
				MsF5Rs	GTGGAGATGTCAATGCAGTGTT		
	MS25	6-FAM	Complex e.g. $GAGA(GAA)(GAAA)_nGA$	MS25F_FAM	TTGGGGTGCTGCCCATTTGC	200-335	240 nM
				MS25R	TCTGGGGACCCCTCAGCGAA		
	MsF33	6-FAM	Complex e.g. $(GACA)_nGAGA(TAGA)_nTGA(TAGA)_n$	MsF33F_FAM	CTTCACAGGGCAGAAGTGG	355-550	120 nM
				MsF33R	GTCAGCTCAAATACGTAGAGATCCA		

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	MsF 31	VIC	Compound e.g. (TGAA)$_n$(TAAA)$_n$	MsF31F_VIC	GTCATACATTTGAGATGGGTAGC	50-145	100 nM
				MsF31R	GTGACTCAGTTGCCAGCTTTG		
	MsF 9	VIC	(TATC)$_n$	MsF9F_VIC	TGGGTGGGAAATAGCTGAAG	148-195	80 nM
				MsF9R	GCCTGTAAACTGCCCAGAGTTG		
	MsF 16	VIC	(CATT)$_n$	MsF16Fs	GCCTTGTCAGTCTGGGTTAGAAC	295-350	140 nM
				MsF16R_VIC	GCTTTATGGTGATAACCAGCACT		
	MsF 3	NED	(ATGA)$_n$	MsF3F_NED	CGTAGGGCTGGTTGGTTTTA	110-190	80 nM
				MsF3R	GCAAGCCTAAGCTGACAAGCA		
	MsF 17	NED	(GATA)$_n$	MsF17F_NED	TGCAATATTGTCATAATTCAACCC	320-400	100 nM
				MsF17R	GACTGATTCACTTGGAGGCCC		
	MsF 18	PET	(GATA)$_n$	MsF18F_PET	AATGGGAAACCATCATGGAA	145-250	280 nM
				MsF18R	GTCAAGTCAAAGTCAGCTTCTGG		
	MsF 28	PET	(TGATC)$_n$	MsF28F_PET	TGACTCAGAACTGTGCCTAATCC	270-320	240 nM

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				MsF28Rsi	CTGTAAATCTCTGGTGTGTGCCT		
	MsF22	PET	$(ATCC)_n$	MsF22F_PET	AGTGGCTGGACCAATGAGAT	220-500	100 nM
				MsF22R	GTTTGCCAAACACAGAGGACC		
Medium							
	MsF5	Quality marker - Details as per Light Multiplex					
	MS3t	6-FAM	Complex e.g. CAAT(GAAA)(GAA)$(GAAA)_n$	MS3F_FAM	GCTGAAGCTGAGAGCTGCATCTGG	170-350	100 nM
				MS3Rt	GTCTCCTCCCCACAACCTAACCCAATCCCC		
	MS4h	6-FAM	$(AAGA)_n$	MS4F	TATTTCATTTTCCCTATCTTCG	420-550	200 nM
				MS4Rh	GCAGTCAACTCAGTAGGGTGTCAG		
	MsF31	Quality marker - Details as per Light Multiplex					
	MS2	VIC	Complex e.g. see figure 4	MS2F_VIC	GCCAGTCAGAGGAGAGCATGGC	170-295	240 nM
				MS2R	TGCTGGCTGTGATGGAAGCTTGT		
	MsF27	VIC	$(ATCT)_n$	MsF27F_VIC	CAAACCCTTTCCCAAATTTCTC	360-500	120 nM
				MsF27R	GCTCATGACCAGCCAGGTCTC		

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	MsF8	NED	(GATG)$_n$	MsF8F	GAGGGCTGAGGATGAATGAA	50-190	50 nM
				MsF8R_NED	AGAACACCAAGGACTCCACG		
	MsF26	NED	(TAGA)$_n$	MsF26F	GAGAAGCCTATGCAATATTCCGGT	300-450	80 nM
				MsF26Rs_NED	GAGGTGATCCAAATCTTACTGTGG		
	MsF4	PET	(CTTT)$_n$	MsF4F_PET	TTGCTTGTCACATTTACAGGG	50-200	120 nM
				MsF4R	GCCTTCCATTGCTCAGTCCTT		
	MS16	PET	Complex e.g. (CAAT)$_n$CAAGAAAGAAAATAG(AAAG)$_n$	MS16F_PET	CAGGTCCAGAAAGAAGGCAG	230-325	100 nM
				MS16R	GTTGGGGACACATAACCTGC		
	MsF22	Quality marker - Details as per Light Multiplex					
Dark							
	MsF5	Quality marker - Details as per Light Multiplex					
	MS27s	6-FAM	Complex e.g. GCAG(GA)$_n$(GAAA)$_n$GGAAGG(AAAG)$_n$(GAAG)$_n$(GAAA)$_n$(AAGA)(GAAA)$_n$(AAGA)$_n$(GAAA)$_n$(GAAAAA)$_n$	MS27Fs_FAM	CCTGTTTACTCTGTTAGATGTGTGGTC	175-300	80 nM
				MS27Rs	GCAAAATCACCAAATTAGCGC		
	MsF24	6-FA	(AACC)$_n$	MsF24F_FAM	CAGCCAAAGCTTTCCATTGT	315-	320 nM

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		M				450	
				MsF24R	GATCAGTGACACATCCCTCCTAGA		
	MsF 31	Quality marker - Details as per Light Multiplex					
	MS9	VIC	(CTTT)$_n$	MS9F_VIC	CAGTGGGCTTGAGATTGAC	150 - 250	250 nM
				MS9R	CATTCCTTAAAACACTCTCAC		
	MsF 14	VIC	(AAGTT)$_n$	MsF14F_VIC	AGGAAGGTTACCAATGAACAAAT	300 - 450	160 nM
				MsF14R	GTCCATGTTGGTTCAGGTTCA		
	MsF 2	NED	(AGAT)$_n$	MsF2F_NED	GGGAGAATCAAACTGTGGCT	50-185	80 nM
				MsF2R	GTTGTGCACATGCAGTGATTC		
	MS17	NED	Complex e.g. (TCCC)$_n$TCCTT(CTTT)$_n$	MS17F_NED	ACCTCGGGTTGGAAATGCACCA	195 - 340	240 nM
				MS17R	TCTGTCCTCGTGGTGGGGGA		
	MsF 15	NED	(TTCTA)$_n$	MsF15F_NED	GGGTGGCATTTAATTTCATTGT	350 - 500	100 nM
				MsF15R	GTATGCTGGGCCCTACAATTC		
	MS13	PET	Complex e.g. (CTTT)$_n$CT(CTTT)$_n$	MS13F_PET	AACAGAGAAGCACAATCACC	50-250	240 nM
				MS13R	TGGCTCTCACTTGATATATTAG		

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	MsF 22	Quality marker - Details as per Light Multiplex					

Table 2: Allele frequencies, observed heterozygosity (H_o) and Polymorphic Information Content (PIC) of 24 STR loci in a reference DNA database of 249 carpet pythons. Minimum and maximum heterozygosities are denoted in bold typeface. Mean heterozygosity is 0.671 (+/- 0.026) and gene diversity is 0.865 (+/- 0.014).Three alleles migrated slightly differently from all other copies of the same allele seen at the three respective loci, suggesting differences in the underlying repeat motif. These alleles are treated as unique for the purpose of the allele frequency database and are included as “alternative alleles” (these were designated absolute base pair integers of 600 for the software analytical stage). Sequencing will reveal underlying polymorphisms in these alleles.

	MsF5	MS25	MsF33	MsF31	MsF9	MsF16	MsF3	MsF17	MsF18	MsF28	MsF22	MS3	MS4h	MsF27s	MsF8	MsF26	MsF4	MS27s	MsF24	MS9	MsF2	MS17	MsF15	MS13	
	N=249	N=248	N=245	N=249	N=249	N=243	N=249	N=249	N=249	N=243	N=248	N=248	N=232	N=244	N=248	N=247	N=248	N=249	N=247	N=244	N=249	N=240	N=233	N=249	
0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.004	
0.2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.002	--	--	--	--	--	
1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.01	
2	--	--	--	--	--	0.00206	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00803	
2.3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00208	--	--	
3	--	--	--	--	--	0.02675	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
3.2	--	--	--	--	--	--	--	--	--	0.00206	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
3.3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00208	--	--	
4	--	--	--	0.34538	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.01606	
4.2	--	0.02621	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00644	0.03213	

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4.3	--	--	--	--	--	--	--	--	--	0.00206	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
5	--	--	--	--	--	0.00412	--	--	--	0.00206	--	--	--	0.0123	--	0.00202	--	--	0.00405	--	--	--	--	0.00602	
5.2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.20683	
5.4	--	--	--	--	--	--	--	--	--	0.00206	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
6	0.01004	--	--	0.03414	--	0.00617	--	--	--	0.00412	--	--	--	--	--	0.01215	0.0504	--	0.02429	--	--	--	--	0.00402	
6.1	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00205	--	--	--	--	--	--	--	--	--	--	
6.2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.1245	
6.3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00405	--	--	--	--	--	
6.4	--	--	--	--	--	--	--	--	--	0.00412	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
7	0.0241	--	--	0.07028	--	0.08436	--	--	--	0.05556	--	--	--	0.00205	0.01008	0.12348	0.01815	--	0.49798	--	--	--	0.01502	0.01606	
7.2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.03614	
7.3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.01205	
7.4	--	--	--	--	--	--	--	--	--	0.00206	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
8	0.00602	--	--	0.07631	0.00201	0.27984	0.01807	0.07831	--	0.0144	--	--	0.00216	0.0082	0.00202	0.00405	0.00403	--	0.15587	--	--	--	0.05365	0.06426	
8.1	--	0.01411	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
8.2	--	--	--	--	--	--	--	0.00201	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.0261	
8.3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00201	
8.4	--	--	--	--	--	--	--	--	--	0.00412	--	--	--	--	--	--	--	--	--	--	--	--	0.00215	--	
9	0.00602	--	--	0.04217	--	0.11317	0.02811	0.01205	0.01004	0.27572	--	--	--	0.0082	--	0.01417	0.00202	--	0.08502	--	--	0.00208	0.08369	0.15863	
9.1	--	0.00806	--	0.00402	--	--	--	0.10643	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
9.2	--	--	--	0.00201	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00602	--	--	--	--	0.00429	0.01606	
9.3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00202	--	--	--	--	--	--	0.00644	0.00402	

ACCEPTED MANUSCRIPT

9.4	--	--	--	--	--	--	--	--	--	0.01235	--	--	--	--	--	--	--	--	--	--	--	--	--	--
10	0.00602	--	--	0.05221	--	0.1893	0.0743	0.00201	0.01004	0.18724	--	--	0.00431	0.0123	0.04637	0.04453	0.00605	--	0.09919	--	--	0.00417	0.09657	0.0502
10.1	0.00201	0.00202	--	0.04016	--	0.00412	--	0.00201	--	--	--	0.00202	--	--	--	--	--	--	--	--	--	--	--	--
10.2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.0241
10.3	--	--	--	0.01606	--	--	--	--	--	--	--	--	--	--	--	0.00405	0.00202	--	--	--	--	--	0.00215	0.00402
10.4	--	--	--	--	--	--	--	--	--	0.00823	--	--	--	--	--	--	--	--	--	--	--	--	--	--
11	0.00803	--	--	0.0261	0.00402	0.1214	0.03213	0.00201	0.00602	0.13374	0.06048	0.00202	0.00216	0.21107	0.09879	0.25506	0.10887	--	0.08704	--	0.00201	0.01667	0.22961	0.01606
11.1	--	0.0121	--	0.01606	--	--	--	--	--	--	--	--	--	0.01639	--	0.00405	--	0.0261	--	--	--	--	0.00644	--
11.2	--	--	--	0.00602	--	--	--	0.00201	--	--	--	--	--	--	--	--	0.00403	0.00201	--	--	--	--	0.00429	0.02811
11.3	--	--	--	0.00602	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00417	0.00644	0.00201
11.4	--	--	--	--	--	--	--	--	--	0.00617	--	--	--	--	--	--	--	--	--	--	--	--	--	--
12	0.06627	--	--	0.0241	0.13253	0.11111	0.06024	0.02811	0.01606	0.18724	0.08468	0.00605	0.01078	0.2623	0.33871	0.12753	0.02016	0.03614	0.03644	0.01434	0.01807	0.02083	0.17382	0.01205
12.1	0.00803	0.00202	--	--	--	--	--	0.00402	--	--	--	--	--	0.02254	--	0.00607	--	0.11044	--	--	--	--	0.00215	--
12.2	--	--	--	--	--	--	--	0.00201	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.01406
12.3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00644	0.00201
12.4	--	--	--	--	--	--	--	--	--	0.00206	--	--	--	--	--	--	--	--	--	--	--	--	--	--
13	0.15462	--	--	0.02811	0.17068	0.04527	0.09438	0.13253	0.06426	0.04527	0.4375	0.03427	0.01293	0.21311	0.29637	0.10729	0.04435	0.03213	0.00405	0.0123	0.01004	0.04375	0.09657	0.01205
13.1	0.00602	0.00403	--	--	--	--	--	0.00201	--	--	--	0.00202	--	0.02049	--	--	--	--	--	--	--	--	--	--
13.2	--	--	--	--	--	--	--	0.00201	--	--	--	--	--	--	--	--	--	0.00402	--	--	--	--	--	0.01205
13.3	--	--	--	--	--	--	--	--	--	--	0.00403	--	--	--	--	--	--	0.09237	--	--	--	--	0.00429	--
14	0.39759	--	--	0.03012	0.21888	0.01235	0.18474	0.10843	0.16064	0.03292	0.16935	0.01815	0.04957	0.09016	0.15927	0.13158	0.07258	0.00201	--	0.03279	0.0241	0.04583	0.08369	0.00201
14.1	0.00602	--	--	--	--	--	--	0.00402	--	--	--	0.00403	--	0.0082	--	0.00405	--	--	--	--	--	--	--	--

ACCEPTED MANUSCRIPT

14.2	--	--	--	--	--	--	--	0.00201	--	--	--	--	0.00216	--	--	--	0.00202	--	--	0.00205	--	0.00208	--	0.04217	
14.3	--	--	0.00408	0.00201	--	--	--	--	--	--	--	--	0.00216	0.00205	--	--	--	0.01004	--	--	--	0.00208	0.00644	--	
15	0.12851	--	--	0.05221	0.21486	--	0.1988	0.09639	0.19679	0.0144	0.08871	0.04435	0.09698	0.04508	0.03226	0.11538	0.08468	0.03213	--	0.09016	0.12048	0.05417	0.0515	0.01004	
15.1	0.00201	--	--	--	--	--	--	0.01205	--	--	--	0.00202	--	0.00205	--	--	--	0.02209	--	--	--	--	--	--	
15.2	--	--	--	--	--	--	--	0.00602	--	--	--	0.00403	--	--	--	--	0.00202	0.01606	--	--	--	--	0.00215	0.00602	
15.3	--	--	0.00204	0.00803	--	--	--	0.00201	--	--	--	--	--	--	--	--	--	0.04819	--	--	--	0.02708	--	--	
16	0.10241	--	--	0.04418	0.15261	--	0.10843	0.15663	0.19076	--	0.06048	0.05242	0.15948	0.02049	0.0121	0.03441	0.09476	0.0241	--	0.16393	0.19277	0.03958	0.02575	0.00201	
16.1	--	0.00202	--	--	--	--	--	0.02811	--	--	--	--	0.00216	--	--	--	--	0.24297	--	--	--	--	--	--	
16.2	--	--	--	--	--	--	--	0.00803	--	--	--	0.00202	--	--	--	--	--	0.00201	--	--	--	0.00833	--	0.00402	
16.3	--	0.00403	0.00612	0.00201	--	--	--	0.00201	--	--	--	--	--	--	--	--	--	0.00402	--	--	--	0.01042	0.00215	--	
17	0.04016	0.00202	0.00204	0.03414	0.07229	--	0.09438	0.07229	0.14056	--	0.04435	0.07661	0.21552	0.0082	0.00403	0.00405	0.11089	--	--	0.1332	0.1506	0.025	0.01288	--	
17.1	0.00402	--	--	--	--	--	--	0.01606	--	--	--	--	--	0.00205	--	--	--	0.05823	--	--	--	--	--	--	
17.2	--	--	--	--	--	--	--	0.00402	--	--	--	--	--	--	--	--	0.01008	--	--	--	--	0.0125	--	--	
17.3	--	--	0.00204	0.00201	--	--	--	--	0.00201	--	--	0.00202	--	--	--	--	--	0.00201	--	--	--	0.01458	0.00429	--	
18	0.00803	0.00202	0.00204	0.0241	0.01807	--	0.04418	0.05422	0.10643	0.00206	0.00605	0.04032	0.23922	0.00615	--	0.00405	0.09073	--	--	0.23156	0.19679	0.00208	0.00429	0.00402	
18.1	--	--	--	--	--	--	--	0.01205	--	--	--	--	--	--	--	--	--	0.00201	--	--	--	--	--	--	
18.2	--	0.00806	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00403	--	--	0.00205	--	0.00208	--	0.00201	
18.3	--	--	0.00612	--	--	--	--	--	--	--	--	0.01411	0.00216	--	--	--	--	0.01205	--	--	--	0.00625	0.00215	--	
19	0.00803	0.0504	0.00204	0.00803	0.01205	--	0.04217	0.02008	0.04418	--	0.03024	0.05847	0.10776	0.00615	--	--	0.07661	0.00602	--	0.19467	0.10442	0.03542	0.00429	--	
19.1	--	--	0.00408	--	--	--	--	0.00201	--	--	--	0.01613	--	--	--	--	--	0.0261	--	--	--	0.00208	--	--	
19.2	--	0.02016	--	--	--	--	--	--	--	--	--	0.00403	--	--	--	--	0.0121	--	--	0.00205	--	--	--	--	
19.3	--	--	0.02041	--	--	--	--	--	--	--	--	0.01815	--	--	--	--	--	0.09036	--	--	--	--	--	--	

ACCEPTED MANUSCRIPT

20	0.00602	0.04839	0.01224	0.00201	0.00201	--	0.01606	0.01004	0.02008	--	0.0121	0.04839	0.04957	0.0041	--	--	0.04637	0.01406	--	0.06148	0.07831	0.02083	--	--	
20.1	--	--	0.00204	--	--	--	--	--	--	--	--	0.01411	--	--	--	--	--	0.01406	--	--	--	0.00833	--	--	
20.2	--	0.03024	--	--	--	--	--	0.00201	--	--	--	0.01411	--	--	--	--	0.00605	--	--	0.0041	--	--	--	--	
20.3	--	0.00605	0.01429	--	--	--	--	--	--	--	--	0.00806	--	--	--	--	--	0.02008	--	--	0.00201	0.00208	--	--	
21	--	0.02016	0.01224	--	--	--	0.00402	0.00201	0.01004	--	0.00202	0.02823	0.02802	--	--	--	0.04234	0.00201	--	0.04508	0.04819	0.01458	--	--	
21.1	--	--	--	--	--	--	--	--	--	--	--	0.01613	--	--	--	--	--	0.01406	--	--	--	--	--	--	
21.2	--	0.03831	0.00204	--	--	--	--	0.00201	--	--	--	0.01008	--	--	--	--	0.0121	--	--	--	--	--	--	--	
21.3	--	0.00403	0.02041	--	--	--	--	--	--	--	--	0.03024	--	0.0041	--	--	--	0.02209	--	--	--	--	--	--	
22	--	0.01815	0.02653	--	--	--	--	--	--	--	--	0.01008	0.00431	--	--	--	0.0121	--	--	0.00615	0.02209	0.0125	--	0.00201	
22.1	--	0.00202	0.00204	--	--	--	--	--	--	--	--	0.02419	--	--	--	--	--	0.00201	--	--	0.00201	0.00208	--	--	
22.2	--	0.0504	0.00204	--	--	--	--	--	--	--	--	0.01411	--	--	--	--	0.01008	0.00201	--	--	--	--	--	0.00201	
22.3	--	0.00605	0.0551	--	--	--	--	--	--	--	--	0.02621	--	--	--	--	--	--	--	--	--	--	--	--	
23	--	0.05645	0.03061	--	--	--	--	--	--	--	--	0.00806	0.00216	--	--	--	0.00202	--	--	0.00205	0.00402	0.00833	--	--	
23.1	--	--	0.00204	--	--	--	--	--	--	--	--	0.02419	--	--	--	--	--	--	--	--	--	--	--	--	
23.2	--	0.05242	0.00816	--	--	--	--	--	--	--	--	0.00806	--	--	--	--	0.00202	--	--	0.00205	--	--	--	--	
23.3	--	0.00806	0.02449	--	--	--	--	--	--	--	--	0.03226	--	0.002	--	--	--	--	--	--	--	--	--	--	
24	--	0.04234	0.01224	--	--	--	--	--	0.00602	--	--	0.01008	0.00216	0.002	--	--	0.00403	--	--	--	0.00402	0.02292	--	--	
24.1	--	--	--	--	--	--	--	--	--	--	--	0.00605	--	--	--	--	--	--	--	--	0.00402	--	--	--	
24.2	--	0.03629	0.00204	--	--	--	--	--	--	--	--	0.01008	--	--	--	--	0.00605	--	--	--	0.00201	--	--	--	
24.3	--	0.00605	0.07551	--	--	--	--	--	--	--	--	0.02016	--	0.002	--	--	--	--	--	--	--	--	--	--	
25	--	0.03427	0.00612	--	--	--	--	--	--	--	--	--	--	0.002	--	--	0.00202	--	--	--	--	0.02292	--	--	
25.1	--	0.00605	0.00408	--	--	--	--	--	--	--	--	0.00403	--	--	--	--	--	--	--	--	0.00201	--	--	--	

ACCEPTED MANUSCRIPT

25.2	--	0.03226	0.01429	--	--	--	--	--	--	--	--	0.02016	--	--	--	--	0.00403	--	--	--	--	--	--	--	
25.3	--	--	0.09592	--	--	--	--	--	--	--	--	0.00605	--	--	--	--	--	--	--	--	--	--	--	--	
26	--	0.03831	0.00816	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00403	--	--	--	0.00402	0.01042	--	--	
26.1	--	0.00202	--	--	--	--	--	--	--	--	--	0.00202	--	--	--	--	--	--	--	--	0.00201	--	--	--	
26.2	--	0.03024	0.02245	--	--	--	--	--	--	--	--	0.00202	--	--	--	--	0.00202	--	--	--	--	0.00208	--	--	
26.3	--	--	0.10612	--	--	--	--	--	--	--	--	0.01008	--	--	--	--	--	--	--	--	--	--	--	--	
27	--	0.04032	0.01224	--	--	--	--	--	0.00402	--	--	0.00403	--	--	--	--	--	--	--	--	0.00201	0.00833	--	--	
27.1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.00202	--	--	--	--	--	--	--	
27.2	--	0.0121	0.02041	--	--	--	--	--	--	--	--	0.01613	--	--	--	--	0.00202	--	--	--	--	0.00417	--	--	
27.3	--	--	0.03878	--	--	--	--	--	--	--	--	0.00202	--	--	--	--	--	--	--	--	--	--	--	--	
28	--	0.02419	0.0102	--	--	--	--	--	--	--	--	--	--	--	--	--	0.01008	--	--	--	0.00201	0.00417	--	--	
28.1	--	0.00202	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
28.2	--	0.01613	0.01837	--	--	--	--	--	--	--	--	0.00605	--	--	--	--	0.00202	--	--	--	--	0.00208	--	--	
28.3	--	--	0.0551	--	--	--	--	--	--	--	--	0.00605	--	--	--	--	--	--	--	--	--	--	--	--	
29	--	0.02016	0.01429	--	--	--	--	--	--	--	--	0.00202	--	--	--	--	0.00403	--	--	--	--	0.00417	--	--	
29.1	--	0.00202	--	--	--	--	--	--	--	--	--	0.00202	--	--	--	--	--	--	--	--	--	--	--	--	
29.2	--	0.01411	0.02041	--	--	--	--	--	--	--	--	0.01411	--	--	--	--	0.00202	--	--	--	--	0.00417	--	--	
29.3	--	0.01411	0.03673	--	--	--	--	--	--	--	--	0.00806	--	--	--	--	--	--	--	--	--	--	--	--	
30	--	0.00806	--	--	--	--	--	--	--	--	--	--	--	0.002	--	--	0.00202	--	--	--	--	0.00833	--	--	
30.1	--	0.02218	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
30.2	--	0.00202	0.01837	--	--	--	--	--	--	--	--	0.0121	--	--	--	--	--	--	--	--	--	0.00417	--	--	
30.3	--	0.0121	0.01633	--	--	--	--	--	--	--	--	0.00806	--	--	--	--	--	--	--	--	--	--	--	--	

ACCEPTED MANUSCRIPT

31	--	0.00202	0.00408	--	--	--	--	--	0.002	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
31.1	--	0.01411	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
31.2	--	0.00403	0.01224	--	--	--	--	--	--	--	--	0.00605	--	--	--	--	--	--	--	--	--	0.0125	--	--	
31.3	--	0.00403	0.00816	--	--	--	--	--	--	--	--	0.00605	--	--	--	--	--	--	--	--	--	--	--	--	
32	--	--	0.00204	--	--	--	--	--	0.004	--	--	--	--	--	--	--	--	--	--	--	--	0.00417	--	--	
32.1	--	0.00806	0.00204	--	--	--	--	--	--	--	--	0.00202	--	--	--	--	--	--	--	--	--	--	--	--	
32.2	--	--	0.00816	--	--	--	--	--	--	--	--	0.00605	--	--	--	--	--	--	--	--	--	0.02292	--	--	
32.3	--	0.0121	0.01429	--	--	--	--	--	--	--	--	0.00403	--	--	--	--	--	--	--	--	--	--	--	--	
33	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	0.01458	--	--	
33.1	--	0.00403	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
33.2	--	--	0.00408	--	--	--	--	--	--	--	--	0.00202	--	--	--	--	--	--	--	--	--	0.03542	--	--	
33.3	--	0.00403	--	--	--	--	--	--	--	--	--	--	0.004	--	--	--	--	--	--	--	--	--	--	--	
34	--	--	--	--	--	--	--	--	0.002	--	--	0.00403	--	--	--	--	--	--	--	--	--	0.02083	--	--	
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36	--	0.00202	--	--	--	--	--	--	--	--	--	0.00202	--	--	--	--	--	--	--	--	--	0.01667	--	--	
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24.2	--	--	0.00408	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
Total alleles	21	67	68	28	11	13	14	36	21	22	12	88	21	29	10	19	42	33	11	17	24	77	29	39	752
Ho	0.618	0.855	**0.865**	0.506	0.703	0.584	0.586	0.663	0.771	0.49	0.609	0.847	0.81	0.68	0.597	0.607	0.738	0.542	**0.397**	0.746	0.759	0.846	0.682	0.61	
PIC	0.7631	0.9683	0.9553	0.8451	0.8075	0.8169	0.8705	0.9012	0.8455	0.8083	0.7318	0.9704	0.8257	0.8083	0.7223	0.8427	0.9258	0.8919	0.6741	0.8309	0.8518	0.9759	0.8664	0.8945a	

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