Evaluation of the use of buccal swabs as an alternative to blood sampling or skin sampling in freshwater turtles for analysis using DArTSeq™

Report to the Australian Reptile Park, Pacific Hwy, Somersby NSW 2250.

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Cover:

Western Sawshell Turtle, *Myuchelys bellii, Roumalla Creek, NSW*. Photo: Arthur Georges.

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EXECUTIVE SUMMARY

An assessment of the effectiveness of buccal swabs for non-invasive sampling of reptiles for DArTSeq™ genotyping (Kilian et al. 2012) was undertaken using the threatened species *Myuchelys bellii* and *Myuchelys purvisi*. The results compared well to those obtained from the traditional approach of blood sampling, and indeed was more successful in that all swab samples yielded adequate DNA, whereas one blood sample failed.

Buccal swab samples are thus an appropriate alternative to other methods of tissue sampling such as collection of blood or skin biopsy, for subsequent analysis using DArTSeq genotyping. For other types sequencing, swabs may not yield sufficient DNA or DNA of sufficient quality, so swabs are seen as complementary to more traditional approaches requiring blood collection or skin biopsy.

Although not evaluated, the effectiveness of swabs rests heavily on following the provided protocols (Figure 2). In particular, attention needs to be paid to swabbing the clean surfaces of soft tissues of the buccal cavity and not relying upon saliva.

It should be noted also that buccal swabs are destructively processed for sequencing, so a single swab will leave nothing for archival purposes. This contrasts with blood sampling or tissue biopsy, where the samples are subsampled for sequencing. To yield comparable outcomes, multiple swabs should be taken from each individual.

A protocol has been provided (Appendix A).

BRIEF

To undertake an assessment of buccal swabs as an alternative to taking blood samples or skin samples from live freshwater turtles, for subsequent analysis using DArTSeq representational genotyping.

VARIATION TO THE BRIEF

None

PREAMBLE

Tissue samples for DNA extraction can be collected from turtles for a variety of purposes, including research, diagnosis of medical conditions, and identification of species. There are several different techniques that can be used to obtain tissue samples from turtles, including biopsy, necropsy and live sampling.

Live sampling involves the collection of tissues from a living turtle without causing any harm or major discomfort to the animal. This can be done by collecting a small amount of blood (Perpiñán 2017). For small turtles, the best sites are the ventral tail vein or the distal scute on the hind leg. For larger turtles, the jugular vein or the brachial vein in the foreleg can be used. Whatever site is chosen, it requires a skilled hand.

Alternatively, a small segment of skin can be taken for DNA extraction. The preferred site of collection is the trailing flap of skin on the vestigial toe of the hind foot, though skin biopsies have been taken from the neck. Sampling the tip of the tail, a common sampling method in lizards, should not be used in turtles. Skin biopsy can carry a small risk of infection, though mark-recapture studies have indicated that turtles recover from these procedures well. Refer to https://www.une.edu.au/__data/assets/pdf_file/0009/246843/W20-Tissue-sampling.pdf for further information.

A less invasive live-sampling technique is to take a buccal swab for the subsequent extraction of DNA (Ghatak et al. 2013). This technique is easily applied by the non-skilled personnel in both the laboratory and the field.

It causes minimal harm and discomfort to the animal, and can be applied to animals of all sizes. Buccal swabbing for DNA sampling from freshwater turtles has been evaluated before (Poschadel and Möller 2004; Thomas et al. 2020) but considered to involve more handling and time to extract the sample compared with blood sampling and was not recommended. The balance between the effort required and the success of sampling will vary with the value placed on the specimens (threatened or endangered) and their body sizes. Buccal swabbing has been used in other reptiles, including lizards, snakes and the Tuatara (Beebee 2008; Schulte et al. 2011) and in amphibians (Pidancier et al. 2003), typically in the context of PCR and/or microsatellite markers.

Here we address a more specific question. How effective is buccal swabbing in comparison with blood collection or skin biopsy for DNA extraction to use for DArTSeq genotyping? We collected paired samples, blood stored on FTA cards and buccal swabs, from the same individuals of two turtle species held by the Australian Reptile Park, NSW, and passed these to Diversity Arrays Technology Pty Ltd for analysis.

Buccal swabbing, following the procedures outlined in this report, provide an alternative to more invasive approaches to obtaining tissue samples for DNA extraction for DArTSeq genotyping and analysis.

SAMPLE COLLECTION

The data were obtained from 11 individuals of *Myuchelys bellii* and 7 individuals of *Myuchelys purvisi* held by the Australian Reptile Park in Gosford NSW. Blood samples were collected from the tail vein of each individual using a fine needle and preserved on Whatman FTA Elution Cards.

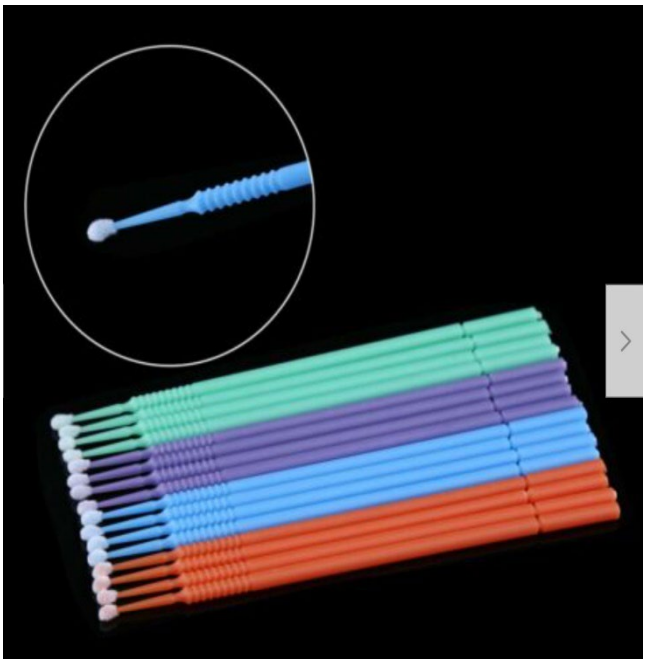

Figure 1. Dental swabs used for buccal sampling of *Myuchelys bellii* and *M. purvisi* (refer Figure 2). The dental swabs were sourced from the internet (https://www.ebay.com.au/str/funnyshopping168)

Buccal samples were collected with dental swabs sourced from China through eBay (Figure 1; https://www.ebay.com.au/str/funnyshopping168). These swabs were chosen because of the discrete collection head, the abrasive nature of the material that made up the collection head (more so than alternatives), and the ease with which the collection head could be removed from the stem. Buccal swabs were stored in 95% ethanol before being shipped to Canberra for analysis.

It is important to note that buccal sampling is not saliva sampling. The head of the swab needs to be gently but definitively passed back and forward over the lining of the buccal cavity to collect shed cells. In this case the tip of the swab was repeatedly moved over the tissue in the roof of the mouth and the tongue. Care was taken to avoid pockets that could contain accumulated food particles or bacterial growth. The swab was placed in 95% ethanol for storage and shipping (Figure 2).

PRELIMINARY ANALYSIS

Data generation

The samples were curated in the Wildlife Tissue Collection at the University of Canberra (UC<Aus>), stored at -20C, and subsampled into a 96 well plate. For the blood samples, a circular disk was punched from the card and placed in the plate for DNA extraction. For the buccal swabs, the tip of the swab (white material in the Figure 3) was separated from the plastic stem and placed directly in the 96 well plate for DNA extraction.

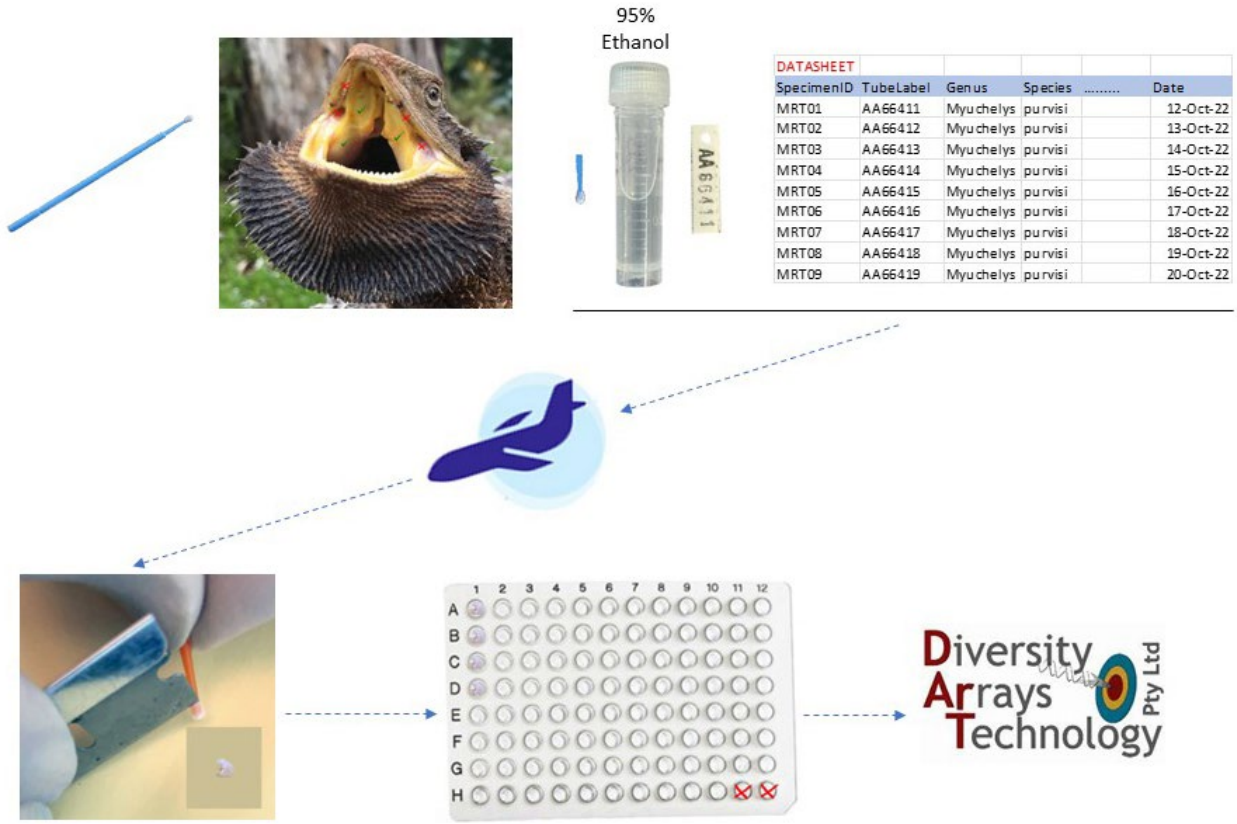

Figure 2. The sampling protocol shown using a rather cooperative bearded dragon. The mouth needs to be opened and kept open during the sampling procedure. The buccal sample is taken by moving the head of the swab gently but definitively back and forward over the lining of the buccal cavity to collect shed cells. Pockets likely to accumulate food material, or bacterial growth (X) should be avoided, as should any buccal parasites. Note that this is not a saliva collection exercise. Once the sample has been collected, the end of the swab is cut off and placed in a labelled tube of 95% ethanol. Care should be taken to avoid any cross-contamination (do not allow swabs to come into contact, sterilize surfaces and equipment between sampling) or other contamination (wear disposable gloves and dispose between specimens). Samples should be packaged in accordance with IATA Packaging Instructions and a copy of Special Exemption A180 should be included with the samples and associated data sheets. If these procedures are followed, Australia Post is a suitable courier.

DNA was extracted by Diversity Arrays Technologies (DArT Pty Ltd, Canberra, Australia -- https://www.diversityarrays.com/) using a NucleoMag 96 Tissue Kit (Macherey-Nagel, Düren, Germany*)* coupled with NucleoMag SEP (Ref. 744900) to allow automated separation of high quality DNA on a Freedom Evo robotic liquid handler (TECAN , Männedorf, Switzerland). Samples were first incubated with proteinase K, adjusted in concentration depending on the sample type.

Sequencing for SNP genotyping was done using DArTseq™ (DArT Pty Ltd) which uses a combination of complexity reduction using restriction enzymes, implicit fragment size selection and next generation sequencing (Kilian et al. 2012; Sansaloni et al. 2011). To achieve the most appropriate complexity reduction (the fraction of the genome represented, controlling average read depth, and number of polymorphic loci),

four combinations of restriction enzymes (PstI enzyme combined with either HpaII, SphI, NspI and MseI) were evaluated and restriction enzyme combination of PstI (recognition sequence 5'-CTGCA|G-3') and SphI (5'-GCATG|C-3') were selected.

DNA samples were processed in digestion/ligation reactions with two different adaptors annealed to the two restriction enzyme overhangs. The PstI-compatible adapter included the Illumina flow cell attachment sequence, a sequencing primer sequence, a barcode region of variable length and the PstI-compatible overhang sequence. The reverse adapter contained flow cell attachment sequence and SphI-compatible overhang sequence. Only fragments generated by the PstI-SphI double digest were effectively amplified in 30 rounds of polymerase chain reaction (PCR). Amplifications consisted of an initial denaturation step of 94°C for 1 min, followed by 30 cycles of PCR with the following temperature profile: denaturation at 94°C for 20 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s, with an additional final extension at 72°C for 7 min. After PCR, equimolar amounts of amplification products from each sample were pooled and applied to c-Bot (Illumina) bridge PCR for sequencing on the Illumina Hiseq2500. The sequencing (single end) was run for 77 cycles.

SNP genotyping

Sequences generated from each lane were processed using proprietary DArT Pty Ltd analytical pipelines. One third of samples were processed twice from DNA, using independent adaptors, to allelic calls as technical replicates, and scoring consistency (repeatability) was used as the main selection criterion for high quality/low error rate markers. The DArT analysis pipelines have been tested against hundreds of controlled crosses to verify mendelian behaviour of the resultant SNPs as part of their commercial operations.

The resultant data were provided in 2-row format as a csv file. These raw data were reconciled with the Wildlife Tissue Collection database and stored in binary format for ease of access.

The SNP data and associated metadata (identity and population) were read into a genlight object (R package {adegenet} on the CRAN repository) (Jombart and Ahmed 2011) to facilitate processing with package dartR (also on CRAN) (Gruber et al. 2018; Mijangos et al. 2022). The raw data had 35,741 loci scored. Average read depth was 10.4*x*. Missed calls occurred at a rate of 8.36%. The raw data are stored in binary format in DFwt22-7436_SNP_raw_data.Rdata. After restricting the samples to those to be used for assessment of swabs vs blood, and removing monomorphic loci, there were 4,925 polymorphic SNP loci scored.

ANALYSIS

Data for *Myuchelys bellii* and *Myuchelys purvisi* were analysed separately. The following is a summary of the results.

a) Genotypes were successfully obtained for all swab samples with a high (96%) call rate for both *M. purvisi* and *M. bellii*.
b) Genotypes were successfully obtained for all but one of the blood samples, with a 95% call rate for *M. purvisi* and a 96% call rate for *M. bellii*. The exceptional individual was ARC013 for which genotyping from blood failed. Both blood and swab samples for ARC013 were eliminated from the analysis.
c) The observed heterozygosity rate was comparable for the swabs and blood for *M. bellii* (at 0.23 in each case) but differed considerably between swabs (0.20) and blood (0.16) for *M. purvisi*. This is thought to reflect poor repeatability in the assignment of heterozygotes by the DArT pipelines arising from the relatively low average read depth (ca 10*x*)

CONCLUSION

Buccal swab samples are an appropriate alternative to other methods of tissue sampling such as collection of blood or skin biopsy, for subsequent analysis using DArTSeq™ genotyping. This conclusion rests heavily

on following the provided protocols, and in particular, paying attention to swabbing the surface of soft tissues of the buccal cavity and not relying upon saliva.

Buccal swab samples are thus an appropriate alternative to other methods of tissue sampling such as collection of blood or skin biopsy, for subsequent analysis using DArTSeq™ genotyping. For other types sequencing, swabs may not yield sufficient DNA or DNA of sufficient quality, so swabs are seen as complementary to more traditional approaches requiring blood collection or skin biopsy.

It should be noted also that buccal swabs are processed for sequencing destructively, so a single swab will leave nothing for archival purposes. This contrasts with blood sampling or tissue biopsy, where the samples are subsampled for sequencing. To yield comparable outcomes, multiple swabs should be taken from each individual.

Acknowlegements

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APPENDIX A

PROTOCOL FOR BUCCAL SWABS

The buccal swab sampling described here is not a saliva test, but rather a means for sampling cheek cells from a suitably exposed buccal surface. One needs to be somewhat vigorous in sampling the cells, though not to the point of causing bleeding.

The Diversity Arrays Technology (DArT) workflows use robotics, and this requires some standardization of quantities. To ensure some level of consistency across swabs and individuals, it is important to saturate the tip of the swab with sample material. Do this by rotating the swab during collection of cheek cells and swabbing vigorously and for a period sufficient to saturate the tip of the swab with cellular material. Failure to do this will result in greater rates of sample drop-out.

Other swabs will work (e.g. cotton swabs), but these present difficulties in that they are not abrasive and the tips are large and it is difficult to achieve consistency across swabs in cell quantity. Please use the swabs provided with the sample kits.

Contamination is an issue in that high levels of contamination can "soak up" the sequencing. Although sequence arising from contamination can be identified and removed, the read depth for target sequence can be compromised. You will get less bang for your buck. So be sure to avoid swabbing in pockets or depressions likely to accumulate food materials or bacteria. Swab bare clean surfaces.

Take duplicate swabs from each animal.

Animals need to be reliably marked for identification preferably using a pit tag. Problems will arise in future services if samples are taken from the same individual at different times, marked with a unique sample number, but not linked reliably to the individual from which they are taken.

Remove the stems and store the swabs in 95% ethanol. Ship using Australia Post making sure to comply with Civil Aviation Safety Authority Special Provision A180.