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REPORT FOR PROJECT: CLOSE-KIN MARK-RECAPTURE (CKMR) TO ASSESS POPULATION ABUNDANCE OF ETP DOLPHINS: PHASE I - SAMPLING FEASIBILITY

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SUMMARY

Following the 2nd Workshop on methods for monitoring the status of eastern Tropical Pacific Ocean dolphin populations, a pilot study was conducted to evaluate the potential for using close-kin mark-recapture (CKMR) methods to assess the population abundances of two species of dolphin in the Eastern Tropical Pacific (ETP). The major goal of the pilot study, also referred to as “Phase I” of the project, was to determine whether dolphin DNA from skin swabs collected via scouring pads could be used for individual genotyping and kinship assignment, which are prerequisites for CKMR. We assessed the quantity and quality of DNA recovered from dolphin skin swabs and genotyped all sampled dolphins (n = 20) at eight microsatellite loci to look for intra- and inter-specific contamination that might hinder individual genotyping efforts. Our results show that high quantities of species-specific, uncontaminated DNA can be recovered from scouring pads, demonstrating that individual genotypes can be obtained from skin swabs using PCR amplification-based methods (e.g., microsatellite genotyping), which allow for identification of parent-offspring pairs. The quality of DNA from skin swabs was variable, however, and substantially lower than the quality of DNA obtained from biopsies. Whether the DNA quality is sufficient for SNP-based genomic approaches that permit identification of half-sibling pairs is yet uncertain, but testing for this is a priority for Phase II of the project. Overall, we are encouraged by the results from Phase I and recommend cautiously proceeding with Phase II.

BACKGROUND

The IATTC provided a total of 120 tissue samples obtained from 20 Eastern Tropical Pacific (ETP) dolphin mortalities from tuna purse-seine fishing operations: 14 spinner dolphins (*Stenella longirostris*) and 6 pantropical spotted dolphins (*Stenella attenuata*). Five tissue samples were collected from each dolphin mortality for DNA analysis (n=100 total): a deep (normal) biopsy as a control and four skin swabs obtained using scouring pads. The biopsy and three skin swabs were taken from each dolphin’s body while it was

on the vessel deck. The fourth swab was taken while the dolphin carcass was still in the water using a protocol closely emulating the in-water sampling protocol that will be used in the future to collect samples for close-kin mark-recapture (CKMR) assessments. In addition, teeth were collected from each dolphin to assist with potential development of an epigenetic clock during Phase II (n=20 total). Methods and Results for the genetic analyses are as follows:

METHODS

Biopsy and skin swab samples stored in 95% ethanol arrived at Nova Southeastern University in good condition. Whether or not there was visible tissue on the scouring pad was recorded for a subset of the swabs (n=46); of these, 74% (n = 34) had clearly visible tissue (Fig. 1), while 26% (n = 12) had no visible tissue. For swabs without visible tissue, regions of the pad with slight discoloration or a circular depression (indicative of attachment to the edge of a pole spear) were assumed to correspond to the dolphin skin contact area and was the pad section from which DNA extraction was attempted.

DNA was extracted from the 20 biopsies using a DNEasy Blood & Tissue Kit (Qiagen, Inc.). However, the same extraction kit produced very low quantities of DNA from skin swabs, so we developed and used a different protocol for the 80 swabs, which entailed tissue lysis with a Qiagen Investigator Lyse & Spin Basket followed by DNA extraction with a Qiagen QIAamp DNA Micro kit. Following DNA extraction, DNA concentration (ng/µl) was measured using a Qubit 3.0 fluorometer (ThermoFisher Scientific) and total DNA quantity in each sample was calculated by multiplying DNA concentration by total elution volume (40 µl for swabs, 150 µl for biopsies). DNA quality (i.e., DNA size and integrity) was assessed using an Agilent Tapestation (Agilent Technologies).

To assess contamination levels caused by potential contact among dolphin individuals, we used previously published DNA primers[1–4] to amplify (via polymerase chain reaction) eight highly variable microsatellite loci in each dolphin of both species. The primers that were selected were confirmed to amplify in both species, which allowed us to detect both intra- and inter-specific dolphin contamination. We compared the microsatellite genotype data obtained from biopsies and in-water skin swabs from each dolphin and looked for any amplified locus showing more than two alleles, which would indicate contamination from another dolphin. Genotype data were generated with an Applied Biosystems 3730 Genetic Analyzer and analyzed using the bioinformatics software Geneious Prime (https://www.geneious.com/).

FIGURE 1: Scouring pad swab sample showing visible dolphin tissue.
FIGURA 1: Muestra obtenida con un estropajo que muestra tejido visible de delfín.

RESULTS AND DISCUSSION

DNA Quantity

The quantity and quality of DNA extracted from biopsies (n = 20) and scouring pad swabs (n = 80) were assessed. Overall, skin swabs produced high quantities of total DNA with our modified extraction protocol (Table 1, Figure 2), confirming that the scouring pads captured DNA-containing tissue. One goal of this assessment was to determine whether we could potentially use DNA from skin swabs for epigenetic aging, which would require total DNA quantities > 10ng per sample. Of the 100 DNA extractions, only four samples had < 100ng total DNA, with just a single sample falling below the 10ng threshold, and this sample was the very first sample extracted prior to protocol modification. Overall, skin swabs produced high quantities of DNA sufficient for downstream genotyping with a variety of methods and, potentially, epigenetic aging.

TABLE 1: Summary statistics of total DNA quantity and DNA concentration obtained from different sample types. Note that biopsy DNA was eluted in 150 µl of buffer, while skin swab DNA was eluted in 40 µl. This is why the mean DNA concentrations are similar but total DNA varies across sample types.
TABLA 1: Estadísticas resumidas de la cantidad de ADN total y la concentración de ADN obtenidas a partir de diferentes tipos de muestras. Cabe señalar que el ADN de las biopsias se eluyó en 150 µl de tampón, mientras que el ADN de las muestras de piel se eluyó en 40 µl. Por este motivo, las concentraciones promedio de ADN son similares, pero la cantidad de ADN total varía según el tipo de muestra.

Sample type	Mean Total DNA Quantity (ng)	Mean DNA concentration (ng/µl)
Biopsy (n=20)	8,001	65.6
In-water swab (n=20)	3,052	73.7

Head swab (n=20)	2,686	63.4
Left flank swab (n=20)	3,186	76.6
Right flank swab (n=20)	2,998	72.2

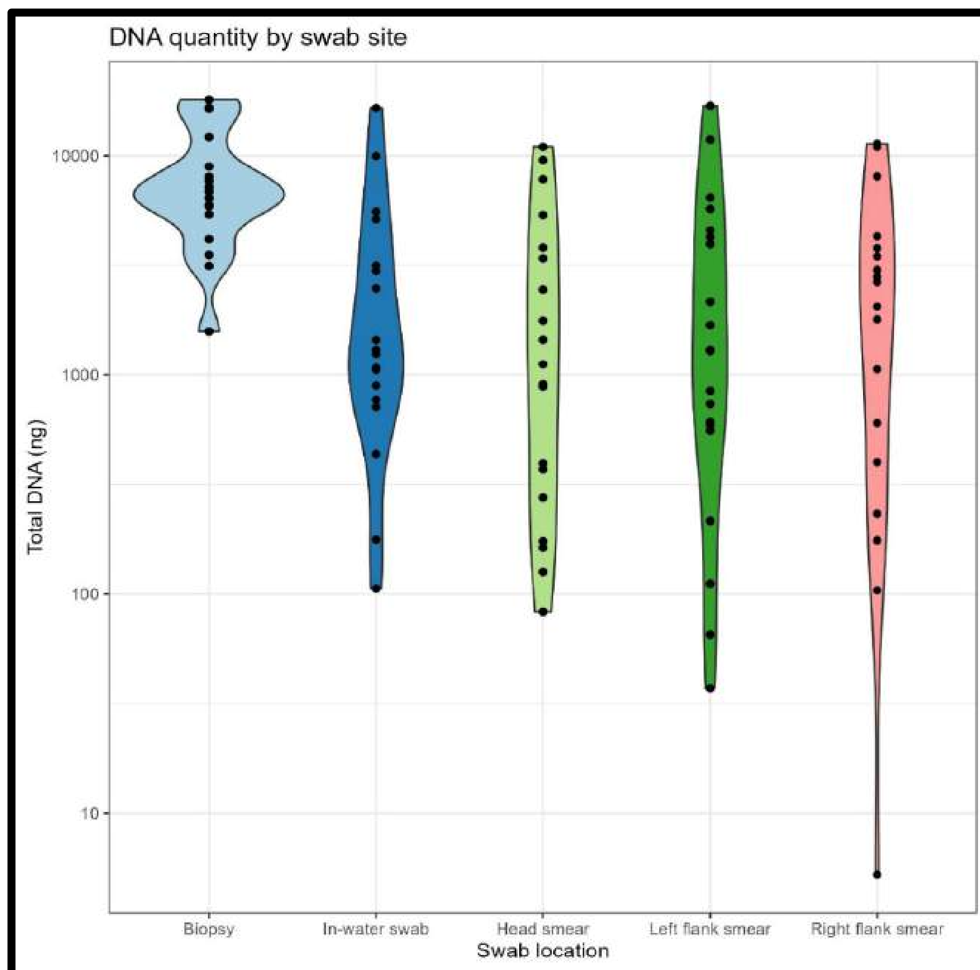


FIGURE 2: Comparative DNA quantity obtained from dolphin biopsies and different skin swab locations with a log-10 transformed Y axis.

FIGURA 2: Cantidad comparativa de ADN obtenida de biopsias de delfines y de diferentes puntos de toma de muestras de piel; el eje 'y' está transformado en log-10.

DNA Quality

High quality DNA contains a high proportion of high molecular weight DNA, which is indicative of minimal degradation. This issue is important for CKMR because genotyping methods that allow identification of second-order relatives (e.g., half-siblings) require high quality DNA, while methods that allow identification of first-order relatives (e.g., parent-offspring) are more lenient about DNA quality. As such, evaluating DNA quality from skin swabs can help calibrate expectations about the kinship categories that will be available for downstream modeling (i.e., can we identify both parent-offspring pairs and half-siblings, or only the former?).

We used an Agilent Tapestation to assess DNA quality. The Tapestation assigns a metric called the DNA Integrity Number (DIN) which scales from 1-10, 1 being highly degraded DNA and 10 being high quality (i.e., high molecular weight) DNA. Among our samples, biopsies consistently produced high quality DNA, while swabs produced lower quality DNA (Fig. 3). Though the DINs were low, most swabs still contained at least some high molecular weight DNA that may still make the samples suitable for genomic approaches that allow identification of half-siblings and second order relatives (see Fig. 4 for three contrasting dolphin sample DNA quality examples). Overall, our results indicate that skin swabs produce variable DNA quality that is neither alarmingly low nor encouragingly high. This may constrain options for downstream genotyping (but see Recommendations section below).

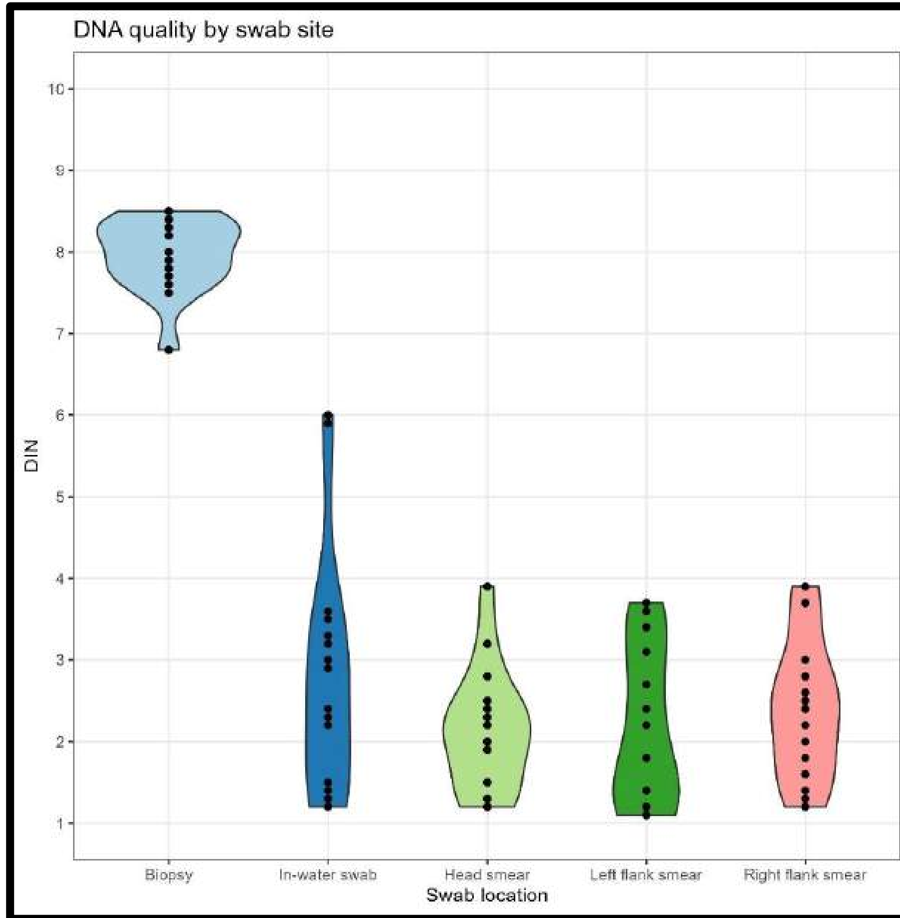
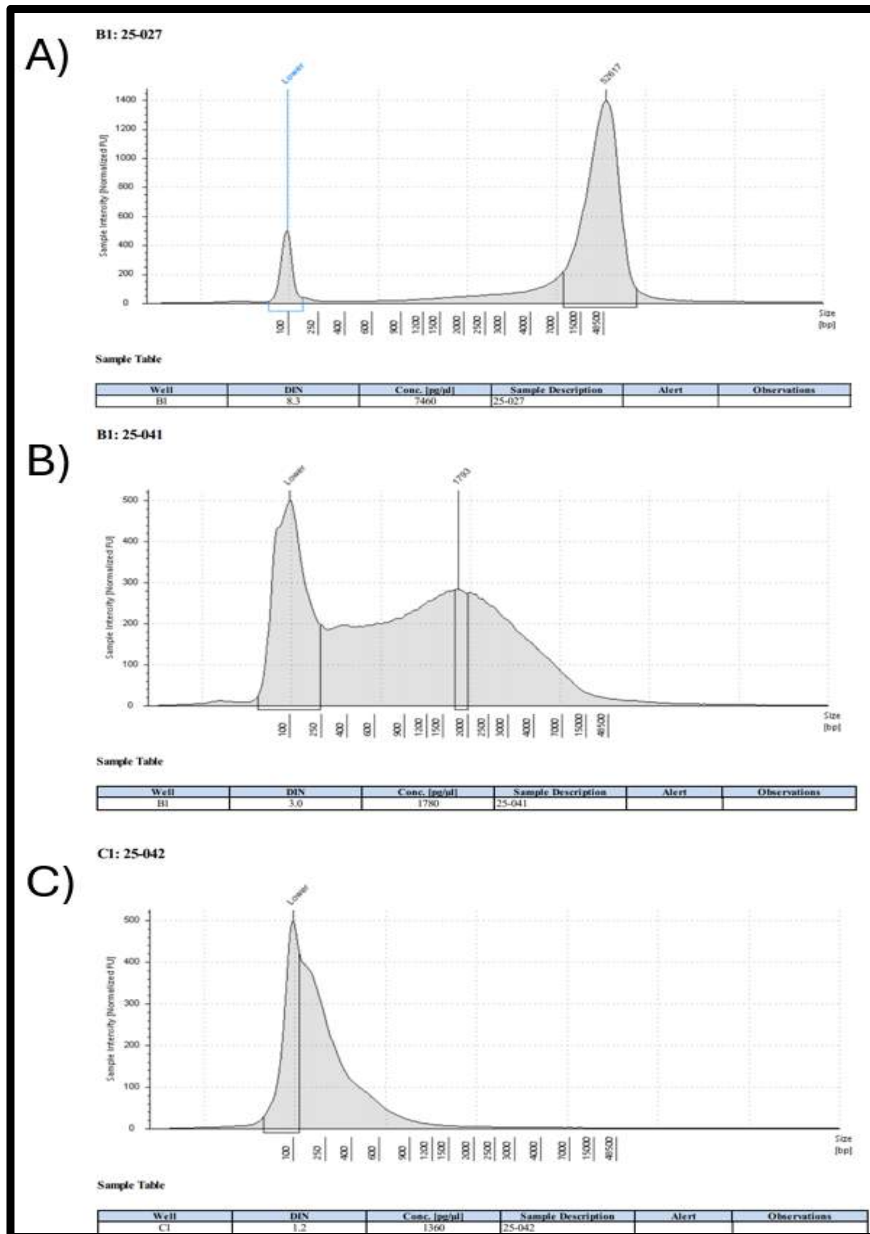


FIGURE 3: Comparative DNA quality (DIN) by tissue sample type and location.
FIGURA 3: Comparación de la calidad del ADN (DIN) según el tipo de muestra de tejido y la ubicación.



Well	DIN	Conc. [pg/µl]	Sample Description	Alert	Observations
B1	8.3	7460	25-027		

Well	DIN	Conc. [pg/µl]	Sample Description	Alert	Observations
B1	3.0	1780	25-041		

Well	DIN	Conc. [pg/µl]	Sample Description	Alert	Observations
C1	1.2	1360	25-042		

FIGURE 4: Three example dolphin DNA quality distribution traces showing high (A), moderate (B), and low (C) quality DNA. The majority of biopsy DNA resembled A, while the majority of swab DNA looked similar to B, with a handful that appeared similar to C. The X axis is DNA fragment length (in base pairs) and the Y axis is fluorescence (a proxy for fragment number). Notice that high quality DNA shows a peak above 15,000 base pairs while low quality DNA is primarily < 1,000 base pairs, while moderate quality DNA is somewhere between the two.

FIGURA 4: Tres ejemplos que muestran la distribución de la calidad del ADN de delfines: alta (A), moderada (B) y baja (C). La mayor parte del ADN de las biopsias se asemejaba a A, mientras que la mayor parte del ADN de las muestras de piel se parecía a B, con unas pocas que se asemejaban a C. El eje 'x' representa la longitud de los fragmentos de ADN (en pares de bases) y el eje 'y', la fluorescencia (un sustituto del número de fragmentos). Cabe destacar que el ADN de alta calidad presenta un pico por encima de los 15,000 pares de bases, mientras que el de baja calidad se sitúa principalmente < 1,000 pares de bases, y el de calidad moderada se encuentra en algún punto intermedio entre ambos.

Intra- and inter-specific contamination

We genotyped eight microsatellite loci from paired biopsies and skin swabs from every sampled individual, using only in-water skin swabs because they most closely emulate samples that will be collected in the future (n = 20 of each tissue type). For each individual dolphin, microsatellite genotypes of biopsies and skin swabs were the same, and no locus/sample combination had > 2 alleles (see Fig. 5 for examples). The consistency of the results combined with the number (eight) of variable microsatellite loci genotyped provides strong evidence that skin swabs contain DNA from the individual swabbed dolphin only. As such, as long as field sampling protocols are consistent, contamination from other dolphins is unlikely to be a concern for this project.

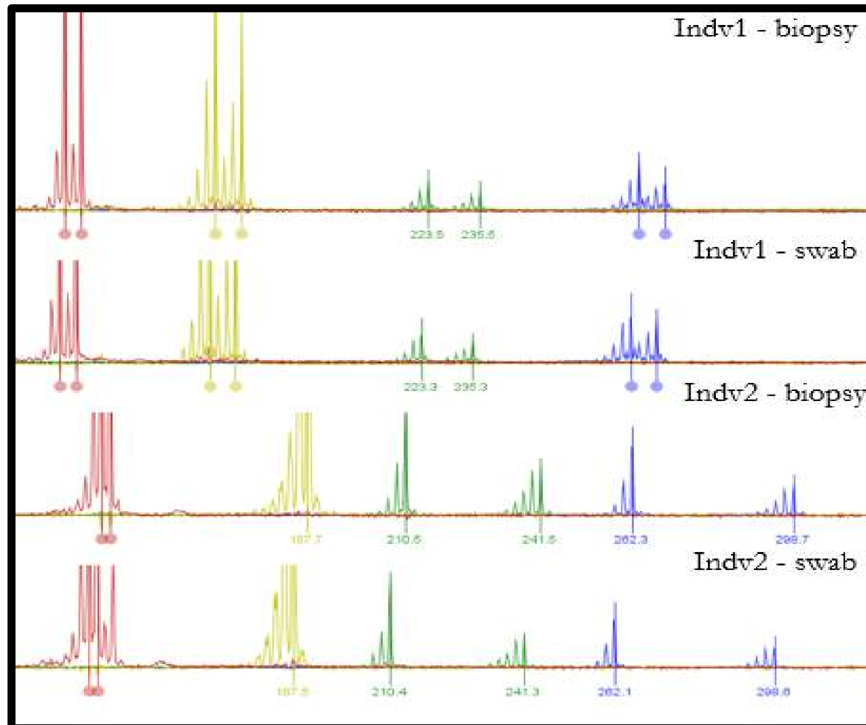


FIGURE 5: Example microsatellite traces of 4 loci from two individual pantropical spotted dolphins. Each color represents a different microsatellite locus. Homozygous individuals show one allele at a locus (e.g. one major yellow peak in Indv2), and heterozygous individuals would have, at most, two alleles (e.g., 2 clearly different purple peaks of different sizes in Indv2). Called alleles are denoted by a number with the fragment size or, if peaks were close together, a circle with a line. All peaks were assigned sizes - the lack of a labeled size in the image is a function of software visualization constraints. The smaller peaks preceding the called alleles are called "stutter" and are expected in microsatellite genotyping data. Note that the biopsies and swabs from each individual show the same genotype for each locus, and note also the size variability in loci between individuals, which suggests that we should be able to detect DNA contamination between dolphin individuals, if it was present.

FIGURA 5: Ejemplos de microsatélites de 4 loci de dos delfines manchados pantropicales. Cada color representa un locus de microsatélites diferente. Los individuos homocigotos muestran un alelo en un locus (por ejemplo, un pico amarillo principal en Indv2), mientras que los individuos heterocigotos tendrían, como máximo, dos alelos (por ejemplo, dos picos morados claramente diferenciados y de distintos tamaños en Indv2). Los alelos identificados se indican mediante un número con el tamaño del fragmento o, si los picos están muy juntos, un círculo con una línea. A todos los picos se les asignaron tamaños; la ausencia de etiquetas con el tamaño en la imagen se debe a limitaciones de visualización del

software. Los picos más pequeños que preceden a los alelos identificados se denominan *"stutter"* y son esperados en los datos de genotipado de microsatélites. Nótese que las biopsias y las muestras de cada individuo muestran el mismo genotipo para cada locus, y nótese también la variabilidad de tamaño en los loci entre individuos, lo que sugiere que deberíamos ser capaces de detectar la contaminación de ADN entre delfines, si la hubiera.

CONCLUSIONS AND RECOMMENDATIONS FOR PROJECT PHASE II

Our genetic results suggest that we can cautiously proceed with Phase II of the dolphin close-kin mark-recapture (CKMR) project. The key remaining molecular questions are whether we can streamline our DNA extraction protocol to accommodate high throughput sample processing and whether the DNA quality of swabs is sufficient for genotyping methods that would permit the identification of second order relatives (e.g., half-sibling pairs). Regarding the former, our current DNA extraction protocol produces high quantities of DNA but is labor-intensive and will be challenging to scale up to process thousands of samples; therefore, as part of Phase II we will explore strategies to automate aspects of the DNA extraction protocol. If the protocol simply cannot be streamlined any further, then plans for the full project will need to account for labor-intensive DNA extractions. One potential way to do this could be to process swabs in small batches in parallel with sample collection. Either way, we do not expect the amount of work to be prohibitive.

Identification of half-sibling pairs requires a minimum of 1,500 - 3,000 informative genetic variants, which can typically only be obtained via genome-wide sequencing methods. Full genome sequencing requires high quality DNA; however, panels that target amplicons containing informative variants (e.g., single nucleotide polymorphisms (SNPs)) have less stringent quality requirements. If we can develop a high-throughput amplicon panel that targets 1,500 – 3,000 SNPs and apply it to skin swabs, we may be able to circumvent constraints associated with DNA quality and ultimately use swab DNA to identify half-siblings.

Our Phase II plans include genome sequencing of DNA from biopsies (which we've shown produce high quality DNA, see Fig. 3) and development of a SNP amplicon panel from those data. The panel will then be tested with DNA derived from skin swabs to confirm its utility with low-moderate DNA quality. If the approach works, then full-scale application of CKMR will be able to leverage both first and second order relatives; if the amplicon panel fails to produce sufficient data from skin swab DNA, then we can still use microsatellites (the method used here to assess contamination) to identify parent-offspring pairs.

Overall, our results justify moving forward with Phase II of the project and provide a roadmap for optimization and testing to ensure that key methodological details are honed prior to deployment of the full project.

ACKNOWLEDGEMENTS

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