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Use of Swabs for Sampling Epithelial Cells for Molecular Genetics Analyses in *Enteroctopus*

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Abstract: We evaluated the efficacy of using swabs to collect cells from the epidermis of octopus as a non-invasive DNA source for classical genetic studies, and demonstrated value of the technique by incorporating it into an effort to determine, within a day, the lineage of captured, live *Enteroctopus* (*E. dofleini* or a cryptic lineage). The cryptic lineage was targeted for captive behavioral and morphological studies, while once genetically identified, the non-target lineage could be more rapidly released back to the wild. We used commercially available sterile foam-tipped swabs and a high-salt preservation buffer to collect and store paired swab and muscle (arm tip) tissue sampled from live *Enteroctopus* collected from Prince William Sound, Alaska. We performed a one-day extraction of DNA from epithelial swab samples and amplification of two diagnostic microsatellite loci to determine the lineage of each of the 21 individuals. Following this rapid lineage assessment, which allowed us to release non-target individuals within a day of laboratory work, we compared paired swab and muscle tissue samples from each individual to assess quantity of DNA yields and consistency of genotyping results, followed by assessment of locus-by-locus reliability of DNA extracts from swabs. Epithelial swabs yielded, on average, lower quantities of DNA (170.32 ± 74.72 (SD) ng/μL) relative to DNA obtained from tissues collected using invasive or destructive techniques (310.95 ± 147.37 (SD) ng/μL. We observed some decrease in yields of DNA from extractions of swab samples conducted 19 and 31 months after initial extractions when samples were stored at room temperature in lysis buffer. All extractions yielded quantities of DNA sufficient to amplify and score all loci, which included fragment data from 10 microsatellite loci (nine polymorphic loci and monomorphic locus EdoμA106), and nucleotide sequence data from a 528 base pair portion of the nuclear octopine dehydrogenase gene. All results from genotyping and sequencing using paired swab and muscle tissue extracts were concordant, and experimental reliability levels for multilocus genotypes generated from swab samples exceeded 97%. This technique is useful for studies in which invasive sampling is not optimal, and in remote field situations since samples can be stored at ambient temperatures for at least 31 months. The use of epithelial swabs is thus a noninvasive technique appropriate for sampling genetic material from live octopuses for use in classical genetic studies as well as supporting experimental and behavioral studies.

Key words*:* cryptic lineage; non-invasive sampling; population genetics; microsatellites*; giant Pacific octopus*

Analysis of DNA for assessment of biodiversity and population genetics studies has become an important component of wildlife and fisheries science and management. Simplified collection and preservation of samples for DNA analysis and inexpensive and reliable DNA extraction methodologies are preferable for population genetics studies of wild species. Non-invasive methods of collecting DNA are increasingly employed and diverse, but typically focus on maintaining the physical integrity of an organism (Baumgardt *et al.* 2013) while providing researchers with a less costly and more efficient way to collect DNA samples. There are currently many protocols for the non-invasive sampling of DNA-containing materials from wild species, particularly vertebrates (Waits and Paetkau 2005, Handel *et al.* 2006, Rodgers and Janečka 2012), but few have been established for marine cephalopods. In the case of octopus (*Enteroctopus*), DNA sampling typically entails clipping the tip of an arm, taking biopsies of other tissues, or collecting whole individuals. Such methods are physically invasive, but may also impact scientific outcome in experimental studies. Lefort *et al.* (2015) recently distinguished the term "non-invasive sampling" from a new term, "non-disruptive DNA sampling," that focuses on the impact of sampling method not only on physical integrity, but also on the fitness and behavior of the individual being sampled. Although it remains unclear whether cephalopods experience pain, suffering and/or distress as a result of arm clipping or other biopsies, Andrews *et al.* (2013, p. 50) concluded that cephalopods possess a suite of attributes "necessary for the ability to suffer pain" and recommended replacement, reduction, and refinement of procedures used when handling cephalopods, in an attempt to decrease stress due to pain. Certainly, physiological responses to pain and discomfort can influence the quality of certain types of scientific data, such as behavioral or physiological data, at least in vertebrates (Hurst and West 2010) and possibly in cephalopods (Andrews *et al.* 2013, Polese *et al.* 2014). In the case of collection of whole individuals, invasive sampling methods can also potentially reduce the number of individuals in the population, and disfigurement from limb clipping may confound assessment of predation or malformations. Thus, in addition to ethical concerns, reduction of adverse effects such as pain and distress is desirable from a scientific viewpoint to avoid confounding experimental results and error in formulating conclusions. As a result, Pidancier *et al.* (2003) recommend that when sampling endangered, vulnerable, or declining species, it is preferable to use the least invasive method; Lefort *et al.* (2015) go further in stating that, ideally, sampling methods would be both non-invasive, and non-disruptive, particularly when applied in support of experimental and behavioral studies.

Population genetic and phylogeography studies have been revolutionized by innovative non-invasive sampling techniques, such as use of buccal cells, hairs, and feathers (Morin and Woodruff 1996, Handel *et al.* 2006). However, relative to extracts from more invasively collected samples, extracts from samples collected non-invasively have been demonstrated to yield lower quantity and often quality of DNA (Handel *et al.* 2006, Sefc *et al.* 2003, Paetkau 2003, Waits and Paetkau 2005, Farley *et al.* 2014), which in turn can introduce new sources of potential error in genetic data gathered from such samples. As a result, researchers continue to recommend a suite of laboratory and analytical guidelines aimed at uncovering and reducing sources of bias and imprecision known to be associated with non-invasively collected samples (Kwok 1990, Woods *et al.* 1999, Paetkau 2003) regardless of the purpose of the study. These include study-specific selection and design of appropriate markers, application of particular quality control procedures such as 'multiple tubes' approaches (Taberlet *et al.* 1996, Gagneux *et al.* 1997), culling of low quality samples using initial screening and analysis techniques and/or comparison of results generated from different tissue sources from the same individual (Paetkau 2003), and application of reliability testing (*e.g.*, Miller *et al.* 2002). The use of buccal swabs for collecting epithelial cell DNA less invasively and less disruptively than collecting blood has become routine in clinical human research, following demonstration of reliability based on analyses of paired (blood, swab) samples (Richards *et al.* 1992, Dickinson *et al.* 2001, Abraham *et al.* 2012), and commercial kits are available. Swabs have also been used to sample epithelial cells for population-level analyses in a number of taxonomic groups: wild birds (buccal cells; Handel *et al.* 2006), amphibians (Pidancier *et al.* 2003, Broquet *et al.* 2007), fish (dorsal epidermis; Livia *et al.* 2006), white abalone (Gruenthal and Burton 2005), and terrestrial slugs (Morinha *et al.* 2014), although quality and reliability of the resulting data, based on paired tissue sampling and/or empirical reliability testing (Miller *et al.* 2002) were not always reported.

Here we report the application of swab-based DNA sampling, storage, and extraction methods, leveraging prior results of population-level sampling and lineage determination of *Enteroctopus* taxa in western Prince William Sound, Alaska. One goal of the study was to test reliability and repeatability of genotypes and sequencing from DNA isolated from epithelial cells collected via swabbing of the epithelium, which would facilitate the use of the technique in general phylogeography and population genetics studies, as well as genetic tagging (Woods *et al.* 1999) efforts. A second goal was to develop a DNA sample collection system that employs a non-invasive and presumably less-disruptive sampling technique that leverages a set of diagnostic genetic markers to assign cephalopod individuals to one of two taxa (Hollenbeck and Scheel 2017. One taxon is the widely-distributed giant Pacific octopus (*Enteroctopus dofleini* Wulker, 1910; hereafter GPO), and the other taxon is a closely allied *Enteroctopus* lineage (hereafter *cryptic*) that may be an undescribed species (Toussaint *et al.* 2012a, Barry *et al.* 2013, Hollenbeck and Scheel 2012, Hollenbeck and Scheel 2017).

The GPO is the most commonly encountered octopus species in the eastern Pacific; it is found from the intertidal zone (Scheel 2002) to depths of 1000 m (Hochberg 1998), and comprises a significant component of by-catch in Alaskan commercial pot fisheries (Reuter *et al.* 2010). The species has been regarded as distinctive in the eastern Pacific due its large size, but in the western Pacific, the GPO belongs to a larger group of common octopuses that may be morphologically difficult to distinguish (Gleadall 1993). The advent of molecular techniques has prompted the reexamination of the taxonomy of north Pacific octopuses (Hochberg 1998, Jorgensen *et al.* 2010). Our prior research (Toussaint *et al.* 2012a), which included data from two mitochondrial genes (cytochrome b and cytochrome oxidase I), three nuclear genes (rhodopsin, octopine dehydrogenase, and paired-box 6), and 18 microsatellite loci, provided evidence of the presence of another, undescribed *Enteroctopus* species (*cryptic*) that is allied to, but genetically divergent from, the GPO in Prince William Sound, Alaska. The existence of this undescribed and previously unrecognized cryptic species raises important questions about the taxonomy of octopus in southcentral Alaska waters, and has implications for determining the impact of commercial pot fisheries on *Enteroctopus* in Alaska (Toussaint *et al.* 2012a). Diagnostic markers, such as those that distinguish between these two *Enteroctopus* lineages (Toussaint *et al.* 2012a), provide a means to design a rapid taxonomic screening test that incorporates the use of epithelial swabs for sampling, aimed at facilitating the selection of individuals to be included in a morphological and behavioral study intended to better understand the taxonomic relationship between the two lineages (Hollenbeck and Scheel 2017).

Here we describe epithelial cell collection procedures, field and laboratory sample storage, and genomic DNA extraction procedures. We demonstrate the applicability of the epithelial swab method in studies of taxonomic diversity in morphological and behavioral studies by using the technique to determine, within a day or two, the lineage of a captured, live *Enteroctopus* (GPO or *cryptic*) held captive on either a research vessel at or near the site of capture, or in holding tanks near the laboratory, so that individuals of the non-target lineage can be released back to the capture site quickly, minimizing stress. Our method, which takes advantage of the presence of two diagnostic microsatellite loci (Toussaint *et al.* 2012a, 2012b, this study) and the use of epithelial swabs instead of muscle tissue from arm tips, provides a less invasive and less expensive sampling procedure, simplifies sample preservation and storage, facilitates a quicker genetic analysis by decreasing extraction time, and validates the epithelial swab method for use in classical genetic studies involving multiple markers and taxon determination.

MATERIALS AND METHODS

Description of problem

We collected live octopuses for short-term morphological and behavioral observation associated with taxonomic studies. We wanted to quickly identify genetic lineages (GPO or *cryptic*) to select individuals for further observation or as museum vouchers, and release the remaining individuals following only a short period in captivity, to minimize trauma and stress. Additionally, we sought to develop a procedure that would minimize trauma for all octopuses under study by avoiding standard tissue sampling procedures. The standard procedure for tissue collection of octopuses for genetic studies has been surgical biopsy, partial arm amputation, or whole animal collection, often during commercial harvest (*e.g.*, Acosta-Jofré *et al.* 2012, but for a recent exception see Larson *et al.* 2015 who obtained samples from deceased animals and shed sucker disks only). We used this opportunity to test whether a less invasive and potentially less disruptive technique for sampling for DNA analysis — collecting epithelial cells via a foam tipped swab — provided adequate results relative to sampling arm tips, so that it could be used in future genetic and behavioral studies of *Enteroctopus*.

Sampling

We collected paired muscle and epithelial cell samples during autumn of 2012, and spring and autumn of 2013. Live octopuses were obtained as by-catch incidental to survey and commercial shrimp fisheries in Prince William Sound (see Hollenbeck and Scheel 2017, for details). Specimens (10 females, 11 males) were collected from depths ranging from 54.9 to 185 meters, and were sampled from various locales in western Prince William Sound (Table 1). Epithelial cells were collected from the same 21 individuals using swabs. For 2012 samples, a single swab was collected per specimen; in 2013, two swabs were collected for each specimen. In the collection of swab samples, each individual was rubbed with a sterile foam-tipped applicator (hereafter called swabs) up or down the mantle for a total of 30 swipes on the dorsal body surface. We used commercially-available sterile swabs (Puritan: Catalog Number 25-1506 1PF 100) that are packaged in individually wrapped sterile paper sleeves. The plastic handle measures 13.5 cm long and the foam tip is an additional 1.5 cm long and 0.5 cm wide. Each swab sample required less than a minute to collect. After epithelial cells were collected, the swab was placed in a 1.7 mL sterile microcentrifuge tube pre-filled with 500 µL Longmire buffer [100mM TRIS pH 8.0, 100mM EDTA, 10M NaCl, 0.5% SDS (w/v); see Longmire *et al.* 1988, Longmire *et al.* 1997]. The swab handle was snipped to a length the tube could accommodate and the lid secured. Arm tip muscle samples were collected from the third left arm (we did not sample the reproductive arm of males) and cut using a sterilized blade or scissors, and stored in 15 mL sterile conical tubes pre-filled with 10mL ethanol (EtOH).

Samples collected for genetic procedures were transported approximately 95 km from Whittier, Alaska, to the USGS Alaska Science Center Molecular Ecology Laboratory (ASC-MEL), located on the Alaska Pacific University (APU) campus in Anchorage, for immediate analyses. Meanwhile, octopuses were held alive, either briefly aboard the research vessel, or transported to the APU aquarium laboratory for captive behavioral and morphological study (Hollenbeck and Scheel 2017), to await the results of the genetic screening. Results of the genetic screen were used to determine which octopuses to release back to the wild as soon as possible and which to hold for further study. Our procedures when handling and sampling from octopuses followed best practices in experimental care for invertebrate animals, and we minimized stress by minimizing handling of animals; however, it took on average less than three minutes handling time to sample an arm tip, more than when sampling the epithelial cells. The research was conducted under Alaska Department of Fish and Game Fish resource permits to D. Scheel and was reviewed for compliance with the Alaska Pacific University Institutional Review Board animal care guidelines and followed the Animal Behavior Society guidelines for the treatment of animals in behavioral research and teaching.

Extraction of genomic DNA

Processing of all swab and arm tip samples commenced within one day of receiving samples in the laboratory at the ASC-MEL, using methods detailed below. Swab samples were processed for use in rapid lineage identification, which included amplification of the DNA at the two diagnostic microsatellite loci. Extraction of DNA from the paired arm tip samples

Table 1. Sampling data and results of lineage identification for each of the 21 *Enteroctopus* samples from Alaska, with allele assignments for the two microsatellite loci (Edoμ A106 and Edoμ A129) used for preliminary lineage assignment and from the nuclear OCDE gene.

Sample ID	Lineage	Sex	Voucher ID CASIZ	Depth (m)	Site Location within Prince William Sound, Alaska	Edoμ A106		Edoμ A129		OCDE alleles	
BRGF12	GPO	F		60.4-142.6	Bald Head Chris Island	126	126	153	153	OCDE1	OCDE1
GBRBF12	GPO	F	190942	54.9-150	Golden Bay, Port Wells	126	126	145	145	OCDE3	OCDE3
RBRF12	*cryptic*	M	190941	60.4-142.6	Bald Head Chris Island	110	110	133	133	OCDE2	OCDE2
RGRF12	*cryptic*	M	192393	60.4-142.6	Long Bay	110	110	127	133	OCDE2	OCDE2
BS13	GPO	M		100	Western Prince William Sound	126	126	147	147	OCDE1	OCDE1
BRBGS13	GPO	F		96	Western Prince William Sound	126	126	145	153	OCDE1	OCDE1
BRGS13	GPO	M		104	Western Prince William Sound	126	126	145	153	OCDE1	OCDE1
RBGRS13	*cryptic*	M	193483	96	Western Prince William Sound	110	110	127	127	OCDE2	OCDE2
RBRS13	GPO	F		100	Western Prince William Sound	126	126	147	147	OCDE1	OCDE1
BBS13	GPO	F		100	Western Prince William Sound	126	126	149	153	OCDE1	OCDE1
GRBS13	GPO	F		100	Western Prince William Sound	126	126	153	153	OCDE1	OCDE1
RBR3S13	GPO	M		100	Western Prince William Sound	126	126	145	147	OCDE1	OCDE1
BBF13	*cryptic*	M		100	Western Prince William Sound	110	110	127	133	OCDE2	OCDE2
BGF13	GPO	M		62-157	Chenega Bay	126	126	145	153	OCDE1	OCDE1
BRGF13	*cryptic*	F	193484	62-166	Herring Bay	110	110	127	133	OCDE2	OCDE2
GBRBF13	GPO	M		64-144	Prince of Wales Passage	126	126	145	155	OCDE1	OCDE1
GRBF13	GPO	M		62-146	Chenega Bay	126	126	153	153	OCDE1	OCDE1
LGF13	*cryptic*	M		57-185	Prince of Wales Passage	110	110	127	133	OCDE2	OCDE2
RBGRF13	GPO	F		62-150	Prince of Wales Passage	126	126	141	153	OCDE1	OCDE1
RBRF13	GPO	F		62-150	Prince of Wales Passage	126	126	139	147	OCDE1	OCDE4
SSF13	*cryptic*	F		62-157	Chenega Bay	110	110	127	133	OCDE2	OCDE2

(hereafter referred to as "whole tissue" samples) commenced simultaneously with extraction from swabs. However, extraction of DNA from muscle tissue using standard techniques is typically of longer duration than for epithelial cells, due to the requirement for mechanical homogenization and longer lysing phase of extraction to disrupt cells in muscle tissue (Dhaliwal and Colette 2013). Thus, rapid lineage determination was not conducted using DNA from arm tips. Following completion of the extraction from the arm tips, a fragment of the octopine dehydrogenase (OCDE) gene was amplified via the polymerase chain reaction (PCR) from both swab and whole tissue samples, and sequenced within a week to verify results of the initial identification; data from the two microsatellite loci were also gathered from the DNA extract obtained from the whole tissue sample. Thereafter, remaining swab samples were stored at room temperature in the laboratory for up to 31 months before being reprocessed. This allowed us to test our ability to obtain DNA from a second extraction from swab samples using the buffer remaining in the original storage tube, and to assess storage longevity. Samples collected in April, May, and October of 2013 were re-extracted after retention at room temperature for eight to 20 months, and then again after 15–27 months, variously. Samples collected in October of 2012 were re-extracted after retention at room temperature for 19 months, then again after 31 months.

Prior to extraction of DNA from swab samples, we rolled the swab around the inner diameter of the storage microcentrifuge tube to dislodge epithelial cells into the Longmire buffer. After pipette mixing, we transferred approximately 150μL of the buffer containing epithelial cells to a clean, sterile microcentrifuge tube for extraction. To extract DNA from whole tissue, we macerated a small portion of the sample using a razor blade and transferred the macerated tissue to clean, sterile microcentrifuge tubes.

We extracted DNA from the 21 paired whole tissue and swab samples collected during 2012 and 2013. To overcome the potential problem of PCR inhibition by mucopolysaccharides present in the skin of octopuses (Cássaro and Dietrich 1977), for this first extraction, we employed the CTAB-PVP (hexadecyltrimethylammoniumbromide/polyvinylpyrrolidone)–chloroform/isoamyl alcohol DNA extraction technique of Stewart and Via (1993), with modifications that included the elimination of ascorbic acid and DIECA (diethyldithiocarbamic acid) from the original protocol. Samples (whole tissues or 150μL of Longmire buffer containing the epithelial cells), were suspended in 850μL of CTAB-PVP lysis buffer (Stewart and Via 1993) to which 0.0035% β-mercaptoethanol and 0.11 mg proteinase K were added and subjected to incubation at 55 °C with agitation for eight hours (for the swab samples) and one to three days (for the whole muscle tissue). The homogenate was extracted with 0.6 volumes of chloroform/isoamyl alcohol (24:1 v/v). Samples were then centrifuged at 16,100 x g for 30 min to separate phases. Following separation, the upper phase (approximately 500–600μL) was transferred to a new sterile microcentrifuge tube. DNA was precipitated with 0.7 volumes of isopropanol for 20 minutes at -80 °C, and centrifuged at 16,100 x g for 30 minutes. Immediately following centrifugation the alcohol was poured off, and the tubes placed upside down on a paper towel. Inverted samples were allowed to dry at room temperature for one to five minutes, after which 500μL of 70% EtOH was added to each tube and centrifuged at 16,100 x g for five minutes. The alcohol was again poured off, and the residue allowed to evaporate completely. The DNA was then suspended in 32μL of 1X TE and quantified using fluorometry as described in Handel *et al.* (2006). Working stocks of each extraction were made by diluting each extraction to 50 ng/μL, and both extractions and working stocks were stored at -20 °C until needed for genetic analyses.

To test another extraction protocol for the swab samples, we also performed a salt extraction, following Medrano *et al.* (1990) with modifications reported in Sonsthagen *et al.* (2004), on the swab re-extractions (performed eight to 20 months after the first extraction). The third extraction was again the CTAB-PVP extraction, performed another 15 (for samples collected in 2013) to 31 months (for samples collected in 2012) later.

Genotyping and sequencing

Rapid lineage determination based on microsatellite genotypes. Among the 18 microsatellite loci found to be polymorphic within GPO (Toussaint *et al.* 2012b), nine are polymorphic in both *Enteroctopus* lineages in Prince William Sound. Although microsatellites are considered imperfect markers for species-level determination, due to the potential for homoplasy and high within-species variation (Estoup *et al.* 2002, Avise 2004), earlier research found that allele spreads at one microsatellite locus (EdoμA129) appeared diagnostic between the GPO [allele sizes: 139–155 base pairs (bp)] and *cryptic* lineage (allele sizes: 127–133 bp) (see Table 7 in Toussaint *et al.* 2012a). Further, one previously unreported locus, EdoμA106, is apparently monomorphic in both lineages, but appears to be fixed for allele 126 in GPO and allele 110 in *cryptic* (Fig. 1A). Laboratory processes associated with genotyping two microsatellite loci are far less time-intensive than obtaining sequence data via Sanger sequencing. Thus, genotyping of these two loci, following short lysis time used for extraction of DNA from epithelial cells, facilitated prompt species determination (within a day of capture) of lineage membership for each individual and assignment of samples to either the GPO or *cryptic* lineage, based on allele sizes for these two loci found after polymerase chain reaction (PCR) amplification of DNA extracted from the swab samples. General genotyping procedures are given in a following section.

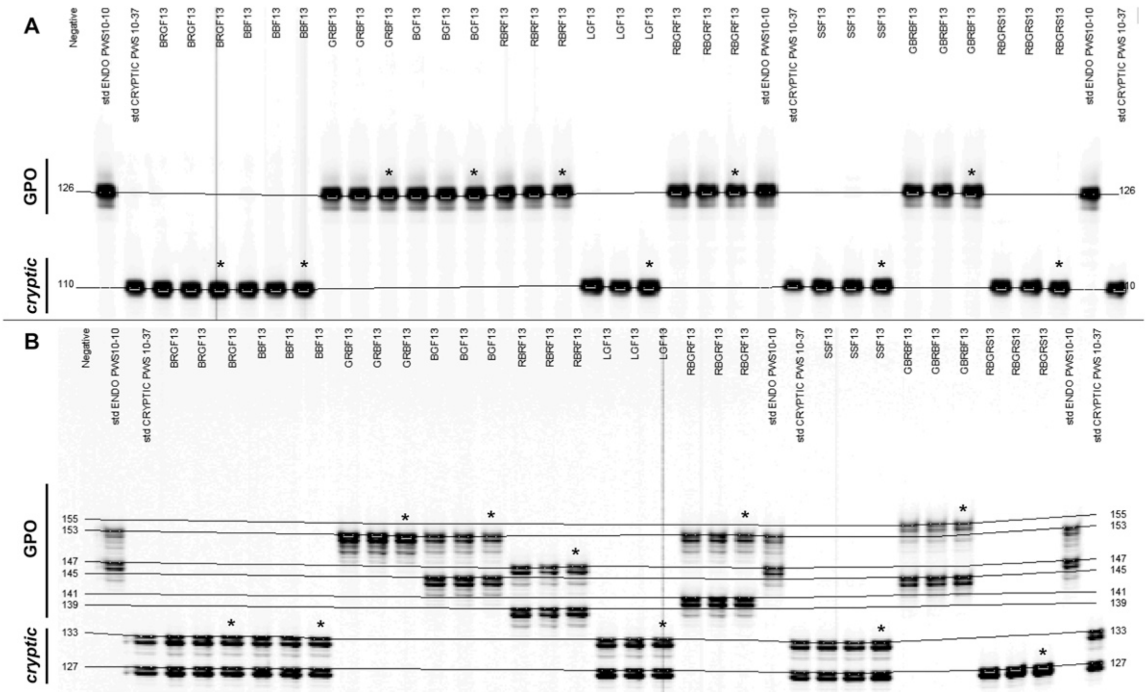

Figure 1. Microsatellite alleles at the A, EdoμA106 and B, EdoμA129 locus from PCR-amplified DNA extracted from 10 paired swab and whole tissue *Enteroctopus* sampled from four locales in Prince William Sound. Samples PWS 10-10 (GPO) and PWS 10-37 (*cryptic*) are previously sized whole tissue extractions included here as standards. A negative control is included in the first lane. Asterisks (*) indicate whole tissue extractions; all other study samples are from extractions of epithelial cells taken from swabs.

Verification of lineage determination based on nuclear nucleotide sequencing. We verified the assignment of each sample to GPO or *cryptic* by comparing sequence data from the nuclear octopine dehydrogenase (OCDE) gene across all paired samples, following PCR amplification and bi-directional sequencing protocols reported in Toussaint *et al.* (2012a). Sequence data were collected several weeks following the preliminary screen via genotyping of microsatellite loci, to independently verify that octopuses retained for morphological and behavioral studies were assigned to the correct *Enteroctopus* lineage using the preliminary lineage determination genotyping method.

Assessment of quantity and quality of DNA extracts from swabs. Quantity of DNA extracts from swabs was assessed by comparing DNA yield (ng/μL) in extractions from paired swab and whole tissue samples. Quality of DNA extracts from swabs was judged by comparing products of the paired swab and whole tissue extracts for 1) genotyping success and levels of allelic dropout in microsatellite loci; and, for paired swab and whole tissue extracts that were sequenced for OCDE (n = 10 pairs), 2) phred quality (Q) scores and base calling error rate (Ewing *et al.* 1998, Ewing and Green 1998), provided by the sequencing alignment package AlignIR 2.0 ™ (LI-COR Inc., Lincoln, NE); and 3) number of reads and manual edits. Q scores quantify the accuracy of sequence data; a score of Q20 for a particular sequence represents the number of base calls in that sequence for which accuracy is 99% or greater. Estimates of genotyping success and assessment of allelic dropout were based on amplification of nine dinucleotide loci (see below).

Following processing for lineage determination, the paired samples (swab and whole tissue) were genotyped at each of the nine dinucleotide microsatellite loci known to be polymorphic in both *Enteroctopus* (see Table 7 in Toussaint *et al.* 2012a). Primer sequences for the nine shared polymorphic loci follow Toussaint *et al.* (2012b); primer sequences for the monomorphic and diagnostic, previously unreported locus (EdoμA106) are as follows: EdoμA106F 5' – CTGGGTTCATTCTCTTGG – 3'; EdoμA106R 5' – TACMCCAATGAGATATGC – 3'. The forward primer for EdoμA106 was tailed on the 5' end with the universal primer sequence M13R (see Toussaint *et al.* 2012b).

PCR amplification and protocols for all genotyping followed Toussaint *et al.* (2012b). We electrophoresed PCR products, which were generated using the 50ng/μL working stocks of extractions, on a 48-well 25-cm 6% polyacrylamide gel using a LI-COR 4200LR automated sequencer (LI-COR, Inc., 1999). Each gel contained two positive standards (one GPO, one *cryptic*) to provide allele sizes; standard sizes were determined via prior reference to an M13 DNA sequence ladder. The standards were used in four to six lanes on each gel (Fig. 1) to size new genotypes using Gene Profiler 4.05 software (Scanalytics, Inc.). All loci were amplified in single reactions (i.e., not multiplexed). For quality control purposes, we reprocessed a minimum of 10% of the samples, randomly selected, for all loci. We also set a priori criteria to re-amplify any sample for which amplicons showed any questionable score, non-amplification of PCR product, extremely weak genotypes, or irregular banding patterns. We observed sterile technique in the handling of all DNA. All procedures were performed with positive and negative controls to provide evidence of replication without contamination.

Genotyping success, allelic dropout, marker resolution and reliability. To estimate genotyping success, we determined consensus multilocus genotypes derived from five amplifications from four swab extractions and the single whole tissue extraction, for the 17 samples collected in 2013. Primers for five of the polymorphic loci assayed in this study (EdoμA8, A105, A122, A125, and E11) amplify fragment sizes greater than 200 bp, and although allelic dropout can occur with any locus, fragment sizes >200 bp have been shown to increase the likelihood of allelic dropout, particularly in suboptimal tissue sources (Sefc *et al.* 2003). Amplification success was determined by calculating the proportion of successful amplifications to total number of amplifications. The rate of allelic dropout was calculated for the nine polymorphic loci (we did not calculate dropout rate for EdoμA106) by determining the ratio of the number of observed allelic dropouts on the number of positive amplification of heterozygous individuals (Broquet and Petit 2004).

To demonstrate the resolution of the multilocus genotypes for application of epithelial swab extractions for genetic tagging studies, we used GIMLET (Valière 2002) to estimate the probability of identity [$P_{(ID)}$] of the nine locus panel. P_{ID} is the probability that two individuals drawn from the same population will have the same n-locus genotype; $P_{(IDsib)}$ provides a conservative upper bound to P_{ID} estimates because it assumes the target population is comprised of first-order relatives (Waits *et al.* 2001). To increase sample size for $P_{(ID)}$ calculations associated with both GPO and *cryptic* lineages in PWS, we augmented the 21-individual dataset with data collected previously from other individuals collected in the region (see Toussaint *et al.* 2012a). We also used RELIOTYPE (Miller *et al.* 2002) to evaluate the reliability of the multilocus genotypes obtained from the swab samples. RELIOTYPE uses a maximum likelihood approach to reduce genotyping errors by providing estimates of dropout probability considering allele frequencies in the parent population. The program provides recommendations for the number of replications required at each locus to reach a >95% reliability level; once individual loci-sample combinations exceeds this level, the data can be considered acceptable without further replication. RELIOTYPE assumes that false alleles do not occur, or can be removed from the data (Miller *et al.* 2002).

Quality of OCDE sequence data was assessed using the phred algorithm (Ewing *et al.* 1998, Ewing and Green 1998) with a mean of 556 high-quality base-pairs per read over the entire dataset, using paired samples from the same sequencing reaction. For the same paired sample comparisons, we also assessed the number of base reads (in total bases scored) and amount of manual editing required for each sequence (in number of base pairs requiring editing).

RESULTS

Rapid lineage determination based on microsatellite genotypes.

Among the 21 samples genotyped using the two diagnostic microsatellite loci (EdoμA106 and EdoμA129), seven were found to belong to the *cryptic* lineage (Table 1). Each octopus was observed in an acrylic aquarium or in a temporary aquarium aboard the research boat prior to release or selection as study subjects based on the results of the rapid genetic screen. Two of the 21 animals died during capture or transportation mishaps; 17 of the others were held alive for a period of one to 14 days. Two *cryptic* lineage octopuses were held alive for 239 and 175 days respectively in the aquarium laboratory to allow additional observations. The former was released alive near the site of capture. The latter, plus three other *cryptic* octopuses and one GPO were vouchered with the California Academy of Science Invertebrate Zoology (CASIZ) (Table I).

Verification of lineage determination based on nuclear nucleotide sequencing.

We observed four alleles among the OCDE gene, two which were previously unreported (OCDE3 and OCDE4). Both novel alleles differed by only one base pair (Fig. 2A) from OCDE1, which was observed by Toussaint *et al.* (2012a) in GPO. There was complete correspondence between the assignment of lineage based on the two diagnostic microsatellite loci (EdoμA106 and EdoμA129) used to rapidly assign lineage, and sequence data from the OCDE gene, gathered several weeks following the preliminary assignment. That is, the OCDE gene associated with the *cryptic* lineage (OCDE2) by Toussaint *et al*. (2012a) was found in all samples that possessed allele 110 at EdoμA106 and allele sizes between 127-133 at locus EdoμA129 (Fig. 1).

Assessment of quantity of DNA extracts from swabs.

We successfully extracted DNA from all 21 samples, and successfully re-extracted these samples after they remained at room temperature for up to 31 months. The average

A

Allele	GenBank No.	Variable Nucleotide Position 114	384	408	443	468	510
OCDE1	KT213460	C	C	T	G	G	T
OCDE2	KT213461	.	T	.	A	A	G
OCDE3	KT225573	.	.	A	.	.	.
OCDE4	KT225574	A	.	.	.	.	.

B

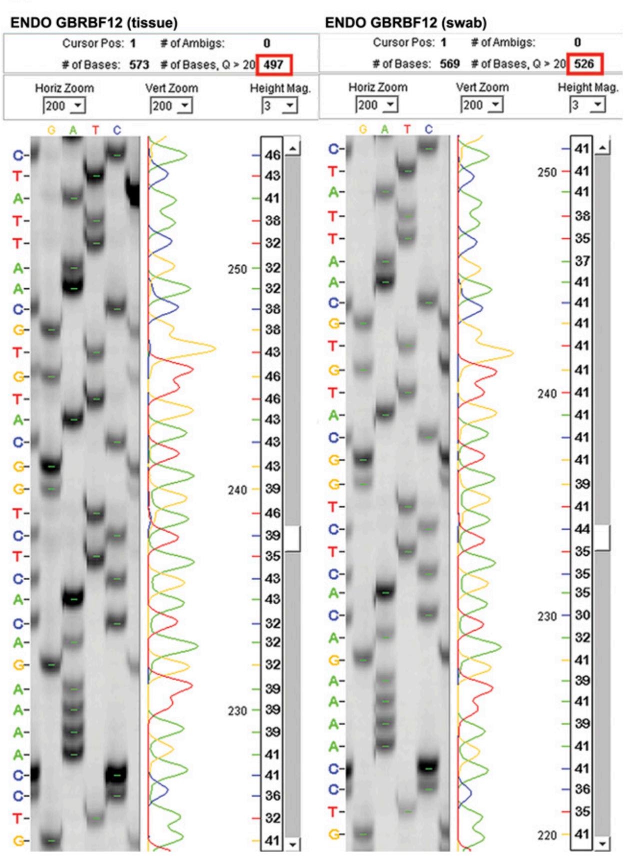

Figure 2. A. Nucleotide sequence variation found in 528bp of the OCDE gene**.** Dots (.) indicate nucleotides that are identical to those of the first sequence. Vertical numbers indicate the position of each variable nucleotide site. Alleles OCDE1, 3, and 4 are found in the GPO lineage; allele OCDE2 is found in the *cryptic* lineage. GenBank accession numbers are associated with a representative sample for the specific haplotype. B. Image from a polyacrylamide gel showing 33 base pairs (bp) from the nuclear octopine dehydrogenase (OCDE) gene of an *Enteroctopus* sequenced from DNA extracted from clipped arm tissue and epithelial cells collected using a swab. Band intensity varied among samples, but in general bands were cleaner in swab extracts. Quality scores (numbers to the right of the electropherogram) were similar to sequence from whole tissue extracts (see text).

fluorometry reading for epithelial swab samples in initial extractions was 170.32 ± 74.72 (SD) ng/μL (range 25 to 430, n = 38); the average fluorometry reading for whole tissue samples was 310.95 ± 147.37 (SD) ng/μL (range 110 to 765ng/μL, n = 21). Swab samples for the first re-extraction (salt) gave fluorometry readings averaging 79.08 ± 27.35 (SD) ng/μL (range 20 to 285, n = 38 swabs); those for the second re-extraction (CTAB-PVP) gave fluorometry readings averaging (78.24 ± 32.33 (SD) ng/μL (range 2 to 157, n = 37 swabs).

Genotyping success, allelic dropout, marker resolution and reliability.

Microsatellite amplicons (Fig. 1) were of sufficiently good quality that multilocus genotypes were recovered from all 21 samples. Of the 21 paired samples, all but one sample successfully amplified alleles at all 10 loci and exhibited clear genotypic results on the initial gel; the exception was BRBGS13, which did not amplify at Edoμ A6 for either whole tissue or swab, even after a second amplification trial (data not shown). There was complete congruence at the 10 microsatellite loci across DNA sources (swab, whole tissue) within species. No allelic dropout was observed in any of the loci assayed via the multiple tubes approach, although the lack of amplification at EdoμA6 in sample BRBGS13 suggests a null at that locus for this specimen. No false alleles were observed across the five amplifications performed for the 17 individuals. Swab samples generally yielded bands of similar intensity as whole tissue samples (Fig. 1). A lack of PCR product in the negative controls, and lack of evidence of co-amplified product (*e.g.*, an observation of more than two alleles at a locus) in product lanes, indicated a lack of cross-contamination or contamination from other sources (human, bacterial, parasitic, etc.).

The $P_{(ID)}$ value for all nine polymorphic loci screened using swabs estimated at $3.539e^{-12}$ for the cryptic lineage, and $6.521e^{-19}$ for the *Enteroctopus dofleini* lineage. The $P_{(IDsib)}$ value was estimated at $4.787e^{-04}$ for the *cryptic* lineage and $7.840e^{-05}$ for the *E. dofleini* lineage. $P_{(ID)}$ and $P_{(IDsib)}$ estimates for both lineages are well within the range recommended for genetic tagging studies (Waits *et al.* 2001). Analyses using RELIOTYPE of swab samples generated experimental reliability levels >97% for the multilocus genotype for each sample. RELIOTYPE recommended the acceptance of 82% of the data without replication (increasing to >88% with one replication for some loci for some homozygous individuals, and to >90% with two replications for some loci for some homozygous individuals). Some individuals had low established reliability for certain loci that were monomorphic for rare alleles (i.e., BRGS13, GRBF13, and RBRF13 for EdoμA6; GRBF13 and BS13 for EdoμA110; and BS13, RBRS13, and GRBF13 for EdoμA129; see Table 2). All microsatellite genotype data are accessioned at the USGS Alaska Science Center data repository (doi: http://dx.doi.org/10.5066/F7VM49HJ).

TABLE 2. Results of RELIOTYPE analysis for *Enteroctopus* samples. The first number in parentheses in each cell indicates the expected number of replicates required to render the genotype sufficiently reliable; the second number indicates the number of replicates rendering the required number of observations per allele with high probability. Asterisks (*) indicate that baseline data did not contain the rare alleles found in those samples and were unable to be analyzed by RELIOTYPE. NP = no product, ER = established reliability, EXP = experimental reliability (after replication), bold indicates accept after initial PCR (ER and EXP = 1.000).

Sample	Lineage	Edoμ A6	Edoμ A8	Edoμ A105	Edoμ A110	Edoμ A118	Edoμ A122	Edoμ A125	Edoμ A129	Edoμ E11	ER	EXP
BRGF12	GPO	*	0(0,0)	0(0,0)	0(0,0)	1(1,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0.9335	0.9803
GBRBF12	GPO	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	1(1,0)	0(0,0)	0(0,0)	0(0,0)	0.9624	0.9802
RBRF12	*cryptic*	1(1,0)	0(0,0)	1(1,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	1(1,0)	0(0,0)	0.9742	0.9953
RGRF12	*cryptic*	0(0,0)	0(0,0)	1(1,0)	1(1,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0.9704	0.9985
BS13	GPO	0(0,0)	0(0,0)	0(0,0)	7(7,7)	0(0,0)	0(0,0)	0(0,0)	6(6,6)	0(0,0)	0.2089	0.9751
BRBGS13	GPO	NP	0(0,0)	0(0,0)	*	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	1.0000	1.0000
BRGS13	GPO	7(7,7)	0(0,0)	1(1,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0.4682	0.9774
RBGRS13	*cryptic*	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	1.0000	1.0000
RBRS13	GPO	0(0,0)	0(0,0)	2(2,0)	0(0,0)	7(7,0)	0(0,0)	0(0,0)	7(7,0)	0(0,0)	0.2855	0.9797
BBS13	GPO	4(4,4)	0(0,0)	1(1,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0.8269	0.9749
GRBS13	GPO	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	1.0000	1.0000
RBR3S13	GPO	4(4,4)	0(0,0)	1(1,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0.8269	0.9749
BBF13	*cryptic*	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	1.0000	1.0000
BGF13	GPO	4(4,4)	0(0,0)	1(1,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0.8269	0.9749
BRGF13	*cryptic*	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	1.0000	1.0000
GBRBF13	GPO	0(0,0)	0(0,0)	1(1,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0.9716	0.9856
GRBF13	GPO	5(5,4)	0(0,0)	2(2,0)	10(10,0)	0(0,0)	0(0,0)	0(0,0)	5(5,0)	0(0,0)	0.0874	0.9755
LGF13	*cryptic*	0(0,0)	0(0,0)	0(0,0)	4(4,3)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0.9160	0.9943
RBGRF13	GPO	*	0(0,0)	1(1,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0.9716	0.9856
RBRF13	GPO	7(6,7)	0(0,0)	2(2,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0.5182	0.9793
SSF13	*cryptic*	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	0(0,0)	1.0000	1.0000

We consistently resolved from 534–612 bp from the OCDE gene (Fig. 2B) from both whole tissue and swabs, but assigned alleles based on only 528 bp (see Fig. 2A for GenBank accession numbers) to facilitate comparison with homologous data from Toussaint *et al.* (2012a). Quality of sequence reads often appeared visually superior, in that we observed less 'background noise' in swabs than in whole tissue extractions (Fig. 2B) and fewer compression artifacts, although sequences from swabs were typically weaker. Comparison of Q scores for the sample pair illustrated in Figure 2B (GBRBF12-swab, Q = 33.92, SD = 7.16; GBRBF12-whole tissue, Q = 32.39, SD = 8.23) showed that in some cases, extracts from swabs did yield superior sequence quality and lower standard deviations than sequence from the paired whole tissue extract. However, there was sample-to-sample variation, and across all 10 paired swab and tissue extracts, Q-scores averaged slightly higher for whole muscle extracts (Q = 34.5, SD = 7.49) than for swabs (Q = 33.32, SD = 7.80). As well, despite the slight increase in Q scores for whole tissue samples over swabs, the quality of sequences from both tissue sources was good; the average Q scores were >30, expected error rate was < 0.001, and base call accuracy was >99.9% for sequences from extracts from both tissue sources. There were small differences in average length of sequence obtained between swab and whole tissue samples, although for the paired samples examined, average read length was slightly higher in whole tissue samples (557 bp) than swab samples (548 bp). On the other hand, the percent of unedited bases with Q scores was slightly higher for swabs (91.6%) than for whole tissue (90.9%). Regardless of these differences, we obtained nucleotide sequence data from the OCDE gene for all 21 samples used in evaluating rates of allelic dropout and false alleles for microsatellite loci. Thus, we were able to obtain data from a minimum of 11 markers from each of the swab samples, and because at least three extractions (30μL remaining following fluorometry assay) were obtained from each swab samples, data can be obtained from at least 90 reactions.

DISCUSSION

Our study demonstrated the value of the swab technique coupled with the use of two diagnostic microsatellite markers to rapidly determine *Enteroctopus* taxonomic lineage as part of an effort to allow more rapid release of non-target lineage live *Enteroctopus* individuals. We also demonstrated that epithelial cells collected by swabbing can be used as a reliable source of genomic DNA for other molecular genetic studies employing classical genetic techniques, in addition to assessment of taxonomic lineage to benefit morphological and behavioral studies aimed at assessing species status (Hollenbeck and Scheel 2017). Quality and quantity of DNA extracts from individual swab samples were sufficient to obtain data using multiple techniques including genotyping of multiple microsatellite loci, ranging in size from 110 to 303 bp, and sequencing of a 612 bp fragment of the nuclear OCDE gene. Although the yield of DNA was on average lower for swabs than for whole tissue samples, we extracted sufficient amounts of DNA in a single extraction to obtain data from a minimum of 11 PCR reactions for each sample with excess DNA remaining for additional processing. Based on our results, we estimate that swab samples will yield data from a minimum of 90 PCR reactions, demonstrating the methods applicability in diverse classical genetics studies, such as genetic tagging, relatedness, population genetics, and phylogeography studies. For example, estimation of $P_{(ID)}$ values for a nine locus microsatellite panel suggests that epithelial cells collected using swabs provide sufficient DNA quantities for reliable use in genetic tagging studies involving either lineage found in Prince William Sound, even if the target population comprises only first-order relatives. For classical sequencing reactions, swabs provided sequence data of equal or higher quality and clarity than whole tissue. Associations of related individuals might occur in species that are direct developers that lack a planktonic stage (*e.g.*, *Octopus bimaculoides* Pickford and McConnaughey, 1949, *O. joubini* Robson, 1929), and possibly among gregarious octopuses (Caldwell *et al.* 2015, Scheel *et al.* 2016). As demonstrated here, the swab technique can also be applied for lineage/species assignment.

Morinha *et al.* (2014) found that average yield from DNA extracted from body swabs taken from terrestrial slugs was 564 ± 314 ng; our yields were much higher, averaging 5,109 ± 2,241.6 ng on the first extraction, although yields were lower than for whole tissue. Morinha *et al.* (2014) stored swabs in sterile empty 1.5mL microcentrifuge tubes and stored them at -20 ºC until DNA extraction. It is possible that increased yields from our swabs were higher due to their immediate placement in a high salt, high EDTA preservation (Longmire) buffer, which allowed our samples to be stored at room temperature throughout the duration of the study. In a study using DNA extracted buccal swabs in amphibians to assay both mitochondrial DNA and nuclear microsatellite loci, Pidancier *et al.* (2003) found yields to be lower in buccal swab samples stored dry for nine weeks at room temperature than in matching buccal samples stored for the same length of time at -18 ºC. Unlike our study, they were unable to obtain nuclear DNA from samples stored at room temperature. Both Pidancier *et al.* (2003) and Handel *et al.* (2006), who used DNA extracted from buccal swabs of nestling birds, speculated that the addition of a buffer solution might depress nucleic acid degradation and reduce bacterial growth in swab samples stored at room temperature. Although our study was not designed specifically to test whether swabs stored in preservation buffer yielded higher quantity and quality of DNA than swabs stored without buffer, we confirmed that the Longmire buffer solution allowed us to store epithelial samples from *Enteroctopus* at room temperature for a long period of time (up to 31 months), with no apparent decrease in amplification success (although average DNA yields decreased over time). We have not yet tested the use of epithelial cells from *Enteroctopus* in genomics (next generation sequencing) procedures.

DNA extracted from swabs yielded identical results at all loci from whole tissue samples, suggesting equal reliability of data collected from swab extracts and whole tissue extracts. We observed no allelic dropout among the microsatellite loci amplified from swabs. It is notable that primers assaying 7 of the 9 polymorphic loci yield fragment sizes which include ranges from 200 to 268 bp. Based on analyses of museum samples, Sefc *et al.* (2003), suggested that microsatellite loci used to perform genetic studies using museum samples (or other samples typically yielding lower quantities of DNA) should target fragment sizes smaller than 200 bp. We therefore suggest a pilot study be performed using the focal species of a new study before committing to using this method, particularly for a genetic tagging study, given the greater impact of error on results of such studies (Paetkau 2003). Although we failed to uncover allelic dropout, even at these seven large loci, we nevertheless recommend the redesign, if possible, of microsatellite primers such that fragment sizes are not larger than 200 bp if they are to be assayed using DNA from poor quality DNA sources. Alternatively, researchers can employ a "multiple tubes" (Navidi *et al.* 1992, Taberlet *et al.* 1996) approach to homozygotes called from loci near or greater than 200 bp.

Other studies that have tested the usefulness of epithelial swabs in genetic studies of mollusks have used commercial kits, such as the QIAamp DNA Micro Kit (Qiagen) for extraction (Morinha *et al.* 2014). The cost of the CTAB-PVP DNA extraction method we used for our initial extraction and second re-extraction, as well as the salt extraction employed for the first re-extraction, is lower than commercially available extraction kits. However, the elapsed time for the CTAB-PVP extraction relative to the Qiagen procedure is longer; further, a number of commercial kits can greatly shorten extraction time, although yields are typically lower (*e.g.*, see Psifidi *et al.* 2015). In a similar study that involved testing the efficacy of buccal swabs in altricial birds, Handel *et al.* (2006), who used the salt extraction we employed in our first re-extraction, noted the longer processing time relative to commercial kits, mainly due to a 12-hour incubation requirement for the method they reported. However, as Handel *et al.* (2006) pointed out, many laboratories have more time than money; thus, the extended incubation time for extractions may not be a large deterrent for using the less expensive method. We also note that although the CTAB-PVP extraction procedure used here typically occurs over a span of approximately one to four days, depending upon the duration of the lysis phase and type of tissue source (*e.g.*, epithelial swabs vs. muscle tissue), actual technician hands-on time is only about seven to eight min per sample, similar to that described using commercial kits (Pidancier *et al.* 2003).

Epithelial swabbing coupled with a high salt field preservation buffer is appealing on several points. Any capture event is stressful to an animal, but minimizing handling will reduce that stress (Williams and Thorne 1996; for octopuses, reviewed in Scheel and Bisson 2012). The epithelial swab technique is less invasive and likely less disruptive, therefore likely to be less stressful to study animals, and minimizes handling time in the field; it can also minimize lethal collection or invasive sampling of non-target species. The procedure is easy to perform and few supplies are needed. Field storage and transport of samples is simple, even in remote areas for long periods, since samples can be stored at ambient temperatures for greater than 31 months without apparent loss of functional DNA quality. The methods presented here provided good DNA yields, reduced processing costs, and appear to be appropriate for both large and small scale studies. Perhaps more importantly, using swabs to sample epithelial cells reduces physical trauma on the sampled octopuses, which is preferable for both scientific and ethical reasons. While we can't determine whether the technique is both non-invasive and non-disruptive, future behavioral studies that use DNA sampling can employ the swab technique and forgo the arm tip amputation technique without sacrificing the quality of resulting genetic data.

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