Métodos em Herpetologia

Procedures for obtaining tissue samples from amphibian and reptile specimens for museomics

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According to current estimates, 41% of amphibians and 21% of reptiles are at risk of extinction (IUCN, 2022), with amphibians being the most threatened vertebrate class due to habitat loss, fragmentation, climate change, and emerging diseases (Kiesecker et al., 2001; Becker et al., 2007; Fisher & Garner, 2020; Luedtke et al., 2023). Unfortunately, a significant portion of species of amphibians and reptiles currently disappeared in nature have not been included in molecular phylogenetic studies, because they were last collected before tissue sampling for PCR amplification and Sanger sequencing (i.e. first generation sequencing) were a common practice (Wandeler et al., 2007; Yeates et al., 2016; Straube et al., 2021).

Amphibians and reptiles in natural history collections are commonly fixed with formalin and stored in 70% ethanol, which can cause damage to DNA through hydrolysis (Lindahl, 1993), accelerate post-mortem DNA damage (e.g. disruption of base-pairing, denaturation, cross-linking between DNA and proteins; Karlsen et al., 1994; Hoffman et al., 2015), and reduce the amount of viable endogenous DNA (Gilbert et al., 2007). As such, procedures to obtain DNA sequences through PCR and Sanger sequencing from wet collections require large amounts of tissue and have a low success rate (Gilbert et al., 2007; Licht et al., 2012). Nevertheless, the adaptation of techniques designed to obtain ancient DNA for paleogenomics and the necessity to obtain historical DNA (hDNA) from museum speci-

mens to answer fundamental questions about biodiversity has given rise to the new field of museomics (Raxworthy & Smith, 2021; Lalueza-Fox, 2022).

The number of herpetological studies employing museomics has increased in recent years (e.g. Hekkala et al. 2011; Kehlmaier et al., 2019, 2021; Lyra et al., 2020; Rancilhac et al. 2020; Scherz et al. 2020, 2022; Ernst et al., 2021; Reyes-Velasco et al. 2021; Vences et al. 2021, 2022; Goutte et al., 2022; Mahony et al., 2022). To date, the two main applications of hDNA in studies of amphibians and reptiles have addressed problems in the identification and phylogenetic position of lost species. For example, Kehlmaier et al. (2019) noted that the identity of multiple subspecies of the chelid turtle *Chelodina mccordi* was unclear based solely on morphology; by analyzing the mitogenomes of specimens from wet collections, they were able to synonymize *C. m. roteensis* and *C. m. mccordi*. Similarly, Lyra et al. (2020) sequenced museum tadpoles of the former *Bokermannohyla claresignata*—which has not been observed in nature for half a century—and found it to be nested within *Boana* as sister of the *Boana pulchella* group. This finding was important to re-evaluate synapomorphies for the group and optimize characters related to suctorial tadpoles and oocyte pigmentation.

The general workflow to obtain hDNA for museomics consists of (1) specimen selection, (2) documentation, (3) material sterilization, (4) tissue sampling, (5) hDNA extraction, (6) library preparation (e.g. single-stranded DNA), and (7) high-throughput sequencing. Protocols for steps 5–7 have been proposed and evaluated in the literature (e.g., Hykin et al., 2015; Straube et al., 2021), but best practices for steps 1–4 have not been established. As such, here we propose brief and simple procedures to obtain tissue samples from specimens of amphibians and reptiles in natural history collections. The workflow is summarized in Fig. 1. We have successfully employed these methods to obtain hDNA from dozens of specimens (Lyra et al., 2020; Straube et al., 2021; unpublished data).

1. SPECIMEN SELECTION. –– The selection of specimens for museomics depends foremost on the scientific question. If the question is related to taxonomic identity, type (holotype, lectotype, or a paratype) or topotypic material is usually required. Alternatively, if the problem is related to the phylogenetic position or population genetics, then any well-preserved specimen can be used. Straube et al. (2021) found no correlation between specimen age and DNA yield, so voucher age should not constrain specimen selection for successful sequencing. Instead, specimen preservation history appears to be

most relevant. As such, we recommend avoiding specimens that are obviously decomposed or otherwise degraded, as they are more likely to yield little or no endogenous DNA. In all cases, specimen selection must be discussed with and approved by the museum curator.

2. DOCUMENTATION.–– Given the risk of cross-contamination Given the risk of cross-contamination (see below), information on each specimen should be carefully recorded in the order in which they are sampled, as this can help identify cross-contamination. Minimally, this entails taking notes on specimen identity (e.g., museum and field voucher numbers and any identifying characteristics, such as collector identity, date, and locality) and tissue source (e.g., muscle obtained via an existing dorsolateral incision). We also recommend photographing each specimen, as well as the jar (showing the label) in which it is stored, as this is useful to confirm specimen identity.

3. MATERIAL STERILIZATION.–– The materials required for tissue sampling are (1) DNA-free gloves, (2) scissors or scalpel, (3) forceps, (4) 1.5 ml Eppendorf tubes, (5) 15 or 50 mL Falcon tubes (or equivalent) (6) ≥ 2% sodium hypochlorite (NaClO; liquid bleach), (7) purified or ultra-purified water, (8) 70% ethanol, and (9) petri dish, kimwipe or paper towel. Depending on specimen size, tissue sampling often must be conducted with the aid of a dissecting microscope. All procedures must be carried out using either latex or nitrile gloves, which must be changed before handling each specimen. Procedures need not be performed in a fume hood or cleanroom lab, but they should not be carried out in a lab where PCR from modern DNA is carried out.

First, fill the three Falcon tubes with sodium hypochlorite, distilled water, and 70% ethanol, respectively (Fig. 2A). Next, before sampling each specimen, thoroughly clean all instruments, ensuring that no residue remains from previous dissections, and sterilize them by immersing in the sodium hypochlorite for 30–60 s, drying with kimwipes or paper towel, immersing three times in the distilled water, drying, and immersing three times in the 70% ethanol and drying thoroughly. Sodium hypochlorite is relevant to disinfect the instrument surface by killing bacteria, fungi, protists, and viruses, reducing the amount of exogenous DNA from contaminants; water is used for removing debris and residuals of sodium hypochlorite from instrument surface; ethanol is also relevant to disinfect the instrument surface by killing remaining contaminants and also denaturing enzymes that could degrade DNA. Alternatively, heat sterilization may also be performed, but this can be difficult when visiting museums because (1) mini high temperature dry sterilizers,

although safe, often are not available in natural history collections, and (2) heat sterilization methods that employ an open flame (e.g. lighting a match, a lighter, or a Bunsen burner) may be prohibited by some curators due to the risk of fire in a building containing jars filled with alcohol.

We recommend using nuclease-free Eppendorf tubes (or equivalent) to prevent DNA degradation (Fulton & Shapiro 2019). Each tube must be filled with 96–100% ethanol, identified, and placed in a rack (Fig. 2B).

Finally, each specimen should be processed separately, in sequence. Preferably the specimen should be washed with clean 70% ethanol before incision. If multiple specimens are stored in the same jar, the selected specimen should be rinsed with distilled water before processing. Position the specimen on a layer of kimwipes or paper towel or a sterilized petri dish, avoiding placing it on contaminated surfaces (e.g., petri dishes of common use in the laboratory). Before using them, petri dishes should be cleaned using sodium hypochlorite, distilled water, and 70% ethanol, following the same sterilization procedure used for the instruments.

4. TISSUE SAMPLING.–– We suggest obtaining a 1–2 mm3 (approximately the size of a small drop of water, ≈10 mg) piece of tissue. We recommend sampling unexposed tissue, either via a new incision or reaching through an existing incision to unexposed tissue, as skin and other exposed tissues are more likely to be contaminated by post-mortem bacterial colonization or human DNA from prior handling. For example, it is common for older frog specimens to have been dissected ventrally to expose the pectoral girdle and viscera, in which case we advise reaching below the skin to obtain unexposed muscle tissue. We recommend sampling muscle because it tends to yield more endogenous DNA than the liver (Lyra et al., 2020; Straube et al., 2021).

DNA from other vouchers stored in the same jar could also serve as a source of contamination (Raxworthy & Smith, 2021), which poses challenges for studies focusing on individual-level biological questions (e.g. identifying specimen identity in cryptic species lacking morphological diagnostic characters, in which all undetermined vouchers are in the same jar). Although FastQ Screen (Wingett & Andrews, 2018) can effectively map unique reads against human and bacterial contaminants for their removal, it might not eliminate reads from contaminant samples of closely related taxa stored in the same jar without also deleting a substantial amount of endogenous DNA from the target specimen. To reduce this risk, it is preferable to collect previously unexposed tissue.

Only researchers who have extensive experience dissecting preserved specimens should perform tissue sampling, especially from type material. Any new incision must be authorized by the curator and should be as small and discrete as possible to facilitate data collection by future researchers. For instance, in anurans, we prefer to perform a short dorsolateral incision on the right side (the stomach is usually on the left) through the skin and underlying muscle to sample trunk muscle (which is both of limited taxonomic value and bilaterally symmetric), as this minimizes damage to the specimen while also enabling visualization of the viscera to confirm sex and maturity and potentially score additional characters. In contrast, taxonomically informative muscles should be avoided (e.g., jaw, thigh, hand, and foot muscles in anurans). Care must be taken to prevent accidentally sampling the digestive tract and inadvertently generating sequences from consumed prey.

In other taxa, tissue selection may vary widely due to morphological diversity and different preservation conditions, but sampling should focus on muscle of limited taxonomic value, as recommended for anurans. For instance, in salamanders and reptiles possessing limbs (e.g. crocodiles, turtles, and many lepidosaurs), a short incision on one of the thighs to access muscles should suffice (e.g. Stuckas et al., 2013). In limbless taxa, like caecilians and snakes, a short paravertebral or ventrolateral incision allows muscle to be sampled. In large specimens, incisions are typically already present along the ventral surface, made during fixation for formalin penetration, thus reducing the need for additional incisions (Ruane & Austin, 2017).

Samples should be stored in 96–100% ethanol at 4°C or, if tissue processing will be delayed, –20°C. Ambient temperature is not recommended, but freezing in an ultrafreezer or liquid nitrogen in a cryogenic tank is not required (though it is also not contraindicated). Repeated freezing/thawing of samples should be avoided to prevent additional DNA damage.

DNA decay seems greater in historical samples from wet collections of anurans than those from bones or dry skin samples of mammals (Sawyer et al., 2012; Straube et al., 2021). However, when good practices are followed in all steps, museomics becomes a powerful tool to obtain reliable molecular data from rare and extinct species. We hope our protocol will enable more researchers to reliably obtain hDNA to address fundamental biological questions.

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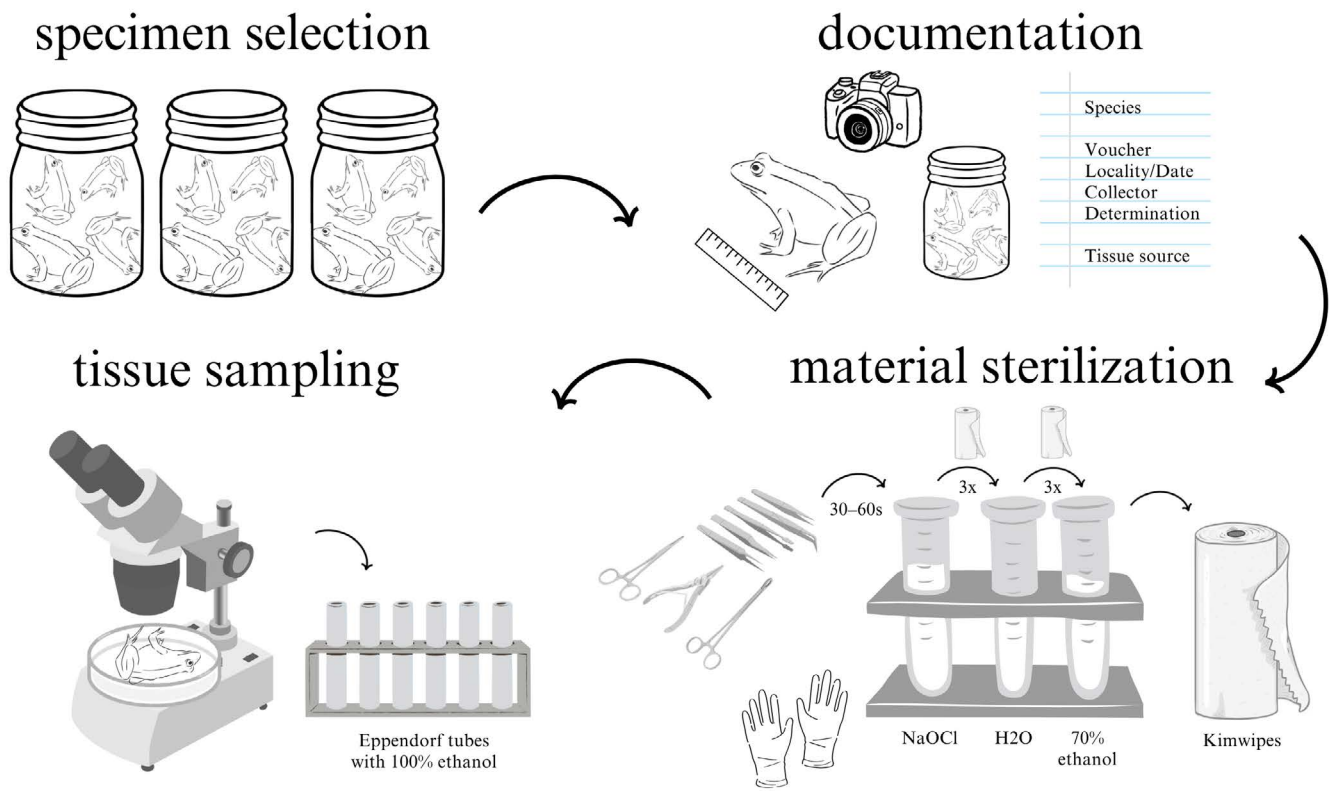

Figure 1. Workflow for collecting tissue samples from amphibians and reptiles for museomics.

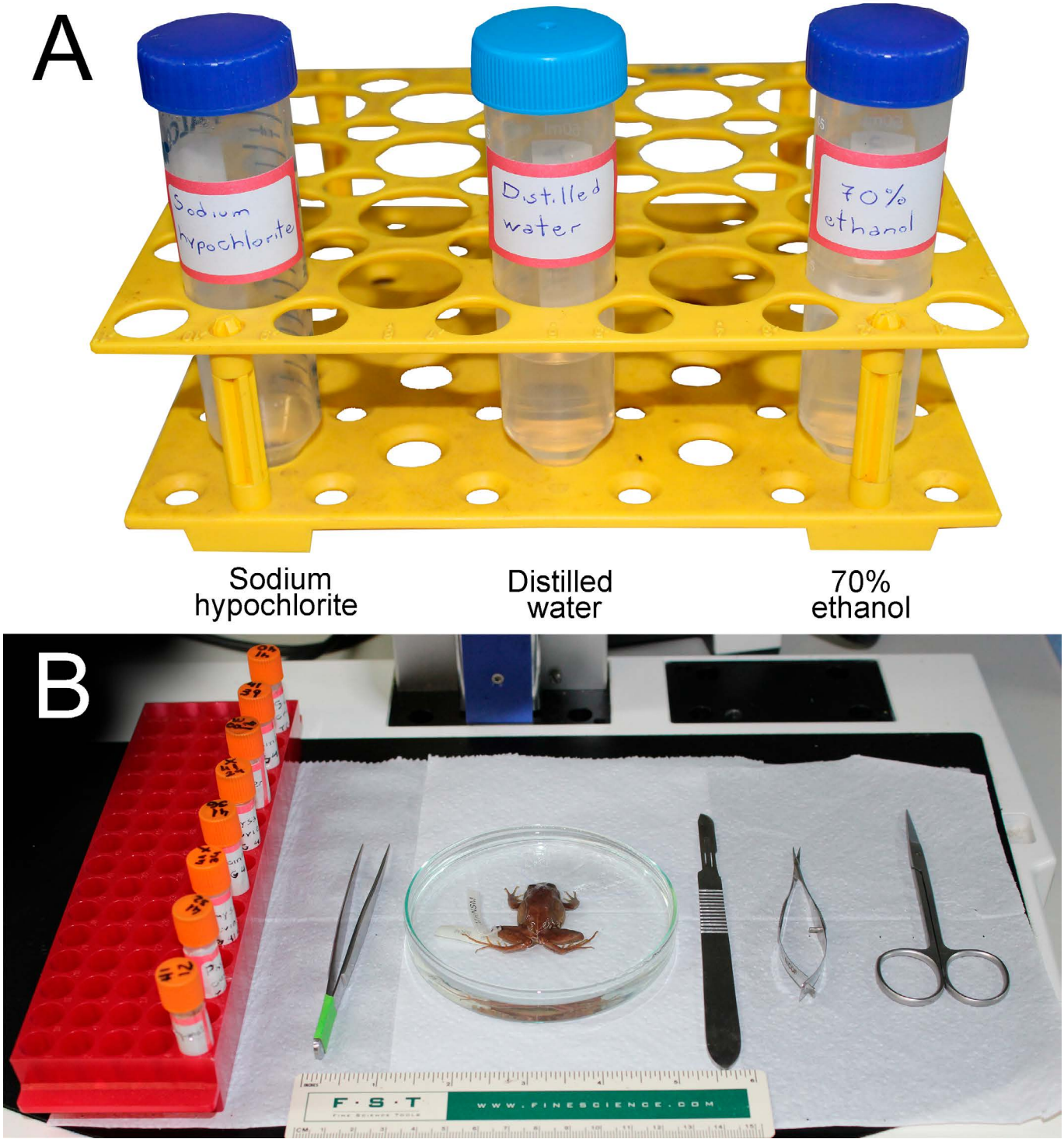

Figure 2. **(A)** Sterilization station with a rack containing three Falcon tubes with ≥ 2% sodium hypochlorite, distilled water, and 70% ethanol. **(B)** Tissue sampling station with a rack containing Eppendorf tubes filled with 96–100% ethanol, a sterilized petri dish, and sterilized materials for microdissection. All procedures must be carried out with gloves. All Eppendorf and Falcon tubes must be identified with sample information (e.g. voucher number) and solution content (sodium hypochlorite, distilled water, and 70% ethanol).