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4/26/13

Joseph A. Cook
Division of Mammals
The Museum of Southwestern Biology at the University of New Mexico

Dear Joe:

I am writing on behalf of my graduate student, Alexis Mychajliw and her collaborator, Nat Clarke, to request the sampling of museum specimens (tissue, skins, skeletons) for DNA extraction for use in our study on the evolution of venom genes within Eulipotyphlan mammals.

Please find included in this request the catalogue numbers of the desired specimens, as well as a summary of the project in which they will be used. We have prioritized the use of frozen or ethanol preserved tissues to avoid the destruction of museum skins, and seek tissue samples from other museums if only skins are available for a species at MSB. The Hadly lab has extensive experience in the non-destructive sampling of specimens for genetic analyses.

Thank you for your consideration and assistance with our research. Please contact Alexis (amychajl@stanford.edu) with any questions or concerns regarding our project or sampling protocols, or for any additional information necessary for your decision and the processing of this request. Alexis is a first-year student in my laboratory at Stanford and her project outline is attached.

As we are located at Stanford University, we are unable to personally pick up loan materials from the MSB. We request that you ship materials to us in ethanol or buffer.

Sincerely yours,

Elizabeth A. Hadly
Paul S. and Billie Achilles Professor of Environmental Biology

Specimens Requested (Tissue – with Barcode, Fluid, Skin, Skeleton/Skull):

Museum of Southwestern Biology, University of New Mexico

Catalogue #	Species	Location	Preparation Type
MSB Mammals 95472	Neomys fodiens	Hungary	Organs (frozen)
MSB Mammals 94681	Neomys anomalus	Hungary	Organs (frozen)
MSB Mammals 151844	Blarina hylophaga	Kansas	Organs (ethanol)
MSB Mammals 95475	Sorex araneus	Hungary	Organs (frozen)
MSB Mammals 229822	Blarina brevicauda	New York	Organs (frozen)
MSB Mammals 48365	Talpa europaea	England	Organs (frozen)
MSB Mammals 47306	Scapanus latimanus anthonyi	Mexico	Tissue (frozen)
MSB Mammals 89528	Sorex monticolus	New Mexico	Organs (frozen)
MSB Mammals 99143	Notiosorex crawfordi	Texas	Organs (frozen)
MSB Mammals 45679	Urotrichus talpoides hondonis	Japan	Organs (frozen)
MSB Mammals 43387	Condylura cristata cristata	Massachusetts	Organs (frozen)
MSB Mammals 148576	Sorex caecutiens	Russia	Organs (frozen)
MSB Mammals 229151	Sorex palustris	Quebec	Tissue (frozen)

Sampling method: DNA extraction from frozen tissue samples or non-destructive tissue extraction from whole organisms in fluid. If no tissue or fluid specimens are available, we request permission to sample museum skins via a skin punch or scrape. If no skin is available, we are able to swab skulls or other bones non-destructively for dried tissue remnants. Alexis will perform these tasks personally while visiting the collections.

PROJECT DESCRIPTION

A. Objectives

The ability to produce and inject venom is widely distributed across the Metazoan phylogeny, appearing basally in cnidarians, such as sea anemones and jellyfish, as well as in a diversity of invertebrate phyla (arthropods, mollusks, etc.) and vertebrates (snakes, lizards, shrews, etc). Venoms are generally a mixture of proteins, salts, polyamines, and/or neurotransmitters, which target key physiological pathways reachable via the circulatory system. Although the morphology enabling venom injection is highly divergent across taxa (spurs, teeth, spines, etc.), the molecular underpinnings of the venom toxins themselves are demonstrated to have resulted from convergent modifications to members of several key protein groups shared across Metazoa.

The protein constituents and genetic underpinnings of many venomous animals have been well studied, particularly in reptiles, spiders, and scorpions due to medical relevance. However, few data yet exist concerning the distribution and evolution of venom in mammals. Current data have been drawn from the known extant venomous mammals (platypus, several shrew species, slow loris, solenodon, and vampire bat – as defined by Fry et al (2009)'s criteria for venom), but represent an incomplete dataset that is too sparse to place in a phylogenetic

context. Morphological and molecular data from known venomous mammals indicates a high degree of divergence between venoms and their venom delivery systems, which strongly suggest at least four independent derivations of venom within Mammalia.

The majority of our knowledge of venom in mammals comes from a recent transcriptome and proteome of the platypus venom gland. The platypus's venom is stored in a crural gland linked to the extratarsal spur that is only present in males, and is used primarily for male-male combat during the mating season. The venom itself contains C-type natriuretic peptides, defensin-like peptides, and nerve growth factors, and produces symptoms of acute pain and swelling. Fossil evidence suggests that the presence of extratarsal spurs is not restricted to platypus, but also found in many multituberculates and other extinct non-eutherian mammals (Hurum et al 2006).

The only venomous primate, the loris (*Nycticebus*), rubs secretions from a modified brachial gland onto its toothcomb prior to biting a potential predator or competitor. As a co-opted social communication mechanism, this venom is derived from immune-related genes and induces anaphylactic shock in bitten humans.

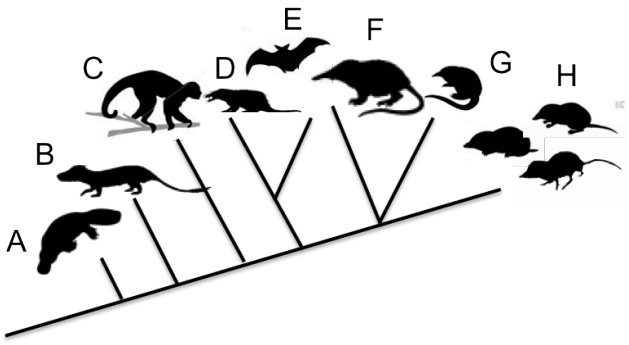

Figure 1: All known venomous mammals. A) Platypus B) Extinct non-eutherian C) Loris D) Extinct Pantolestid E) Vampire Bat F) Solenodon G) Extinct Nesophontid H) Soricinae: Blarina, Neomys, 2 Extinct Shrews

Most venomous mammals are concentrated in the Laurasiatherian clade. A fossil pantolestid from the Paleocene, *Bisonalveus browni* (Fox & Scott 2005) is the earliest eutherian mammal with tooth grooves suggestive of an envenomation apparatus. Although both venomous bats (vampire bats of Desmodontinae) and venomous Eulipotyphlans (solenodons, shrews) have an oral envenomation method and modified salivary glands, their uses and co-opted genes are entirely distinct. Vampire bats use specialized teeth to pierce prey's skin, and the anticoagulant saliva primarily relies on modified plasminogen activators to keep blood flowing while the bat feeds. It appears that this ability evolved once within the subfamily.

Conversely, Eulipotyphlans have a modified salivary gland, which produces saliva that itself is toxic and used to immobilize prey items. LD50 values (median lethal dose, dose that kills 50% of population) have been ascertained for the saliva of the Hispaniolan solenodon, *Solenodon paradoxus*, the Northern short-tailed shrew, *Blarina brevicauda*, and the European water shrew, *Neomys fodiens,* on mice and rabbits (Dufton 1992). Solenodons have a deep tubular groove on their second lower caniniform incisor that conducts venom directly from the gland into a bitten prey item. *Blarina* and *Neomys* lack a well-defined groove, but instead have a shallow concavity on the lingual surface of their enlarged lower incisor that allows for the collection of saliva prior to injection.

Three fossil taxa within Eulipotyphla have morphological features suggesting the presence of venom. The fossil group Nesophontidae is endemic to the West Indies, co-occurring with solenodons. All species examined have upper canines with well-defined longitudinal grooves (Turvey 2010), which strongly resemble the grooves of solenodon incisors. The Pleistocene fossil shrews *Beremendia fissidens* and *Dolinasorex glyphodon* (Rofes & Cuenca-Bescos 2009), placed within Soricinae (red-toothed shrews, the subfamily that contains *Blarina* and *Neomys*), have similar gutter-like grooves on their lower incisors.

The Cuban solenodon, *Solenodon cubanus,* is known to be venomous due to symptoms displayed by bitten researchers that are highly similar to those produced by *S. paradoxus*, as well as the presence of a homologous groove on the second incisor. The European hedgehog, *Erinaceus europaeus*, was once suspected to be venomous due to behavioral observations, but subsequent tests of their saliva injected in mice yielded no evidence of venom. Other species that are thought to be venomous due to natural history accounts include *Crocidura canariensis*, *Talpa europaeaus, Sorex araneus,* and *Sorex palustris*.

The main functional protein of *B. brevicauda* venom secretions, known as Blarina toxin (BLTX; Genbank AB111919), has been isolated and characterized, along with two other salivary proteins in the same protein family of kallikrein proteases as BLTX (Genbank AB105056, AB105055; Aminetzch et al 2009). BLTX shares convergent structural modifications with the kallikrein-based toxin of beaded lizards, which are found nowhere else within sequenced mammals. Aminetzach et al (2009) were able to predict structural modifications that would transform the kallikrein gene of a nontoxic species to one with toxic functions. We will use this available genetic sequence data to amplify kallikrein genes of venomous and nonvenomous Eulipotyphlan mammals.

For this project, we seek to address a nested set of hypotheses regarding venom gene evolution within Eulipotyphla, and its implications for understanding morphological evolution of envenomation mechanisms and clade diversification rates. We hypothesize that venom is (1) basal to the Eulipotyphlan clade; (2) convergent within Eulipotyphla, as basal to Soricinae and basal to Solenodontidae; (3) evolved independently in *Blarina, Neomys*, and *Solenodon*. Many of the species within a venomous genus have not been tested for venom either in the lab or in natural history observations. Our project will provide the springboard for numerous additional behavioral, morphological, and genetic studies of confirmed venomous or potentially venomous species as described by our protein modeling.

Our specimen selection criteria is as follows:

1) Is it known to be venomous?
2) Is it hypothesized to be venomous based on natural history or personal accounts?
3) Is it within a genus that contains at least one confirmed venomous species?
4) Does it have traits that mimic venomous species, such as a semi-aquatic lifestyle or hoarding of prey items?
5) Additional samples to ensure balanced random sampling of phylogeny, for purposes of gene tree and species tree reconstructions

B. Time Frame

We have begun designing primers for amplifying kallikrein genes, and identified all taxa for this project. Additionally, we have compiled a database of relevant ecological characters such as body size for all species selected. We are prepared to begin DNA extractions and amplifications as soon as we receive tissue samples.

We anticipate that all genetic lab work will be completed by July 2013. Analyses will continue through September 2013. We expect to complete this project and a first draft manuscript by January 2014.

C. Availability

Although a small number of species selected are native to California (where we are located), we do not intend to procure samples from the wild. This is because our work is in a purely phylogenetic context and we require samples from myriad places across the world. Furthermore, as we are only interested in a small number of genes that are likely monomorphic, we need only one individual per species and do not require information on location, year collected, or other specifics that may be useful in a phylogeographic or population-level study.

The full genomes of *Sorex araneus* and *Erinaceus europaeus* are available online, but at very low coverage. We request tissue specimens of these species to allow for cross-validation with our computational searches for kallikrein genes within these genomes.

We prioritize sampling from Berkeley's Museum of Vertebrate Zoology due to its proximity to Stanford University, allowing us to personally visit the collections and assist in sampling. For tissues not available there, we next ask the Museum of Southwest Biology in New Mexico. Lastly, for very rare species, we request specimens from the American Museum of Natural History.

D. Methods of Analysis

We will extract DNA using a standard Qiagen DNAeasy Blood & Tissue Kit from samples either frozen or from ethanol. The organ or source of tissue is irrelevant for our purposes. Thus, when sampling from a whole organism in ethanol, we are able to take a very small tissue sample from an inconspicuous part of the body that will not compromise the specimen for further analyses, morphological or otherwise.

For those specimens without tissue or ethanol available, Alexis will personally visit the AMNH collections and take samples from skins or skeletons/skulls. Standard procedures for obtaining DNA from museum specimens will be followed (ie Casas-Marce et al 2010) to

minimize negative impacts to specimens. As the Hadly lab is specialized in acquiring DNA from ancient sources, we expect a high success rate for any DNA obtained in this way.

We will amplify the DNA using primers designed from sequences of shrew kallikrein genes (as described previously). We will also amplify select mitochondrial and nuclear loci, based on availability from GenBank, to allow us to perform independent phylogenetic reconstructions to compare with the standard mammalian supertree from Bininda-Emonds et al (2007), which has many polytomies associated with shrew species (the entire *Crocidura* clade is a giant polytomy, for example). This is important, as we will use branch lengths and divergence times for further analyses.

E. Qualifications

The primary author, Alexis Mychajliw, has experience working both in museum collections and in genetics labs. She has successfully carried out DNA sequencing projects for studies of rodent population genetics and phylogeography. She is well versed in all requisite phylogenetics software and relevant morphological analyses. This project will form a portion of her dissertation focusing on Eulipotyphlan mammals, which includes future fieldwork to sample wild *Solenodon* populations.

The second author, Nat Clarke, has research experience in molecular biology, protein biochemistry, genomics, and sequence analysis, as well as museum research experience. He has performed similar protein modeling studies and has research expertise in the venoms and venom delivery systems of reptiles.

Our faculty advisor for this project, Dr. Hadly, has experience ranging from population genetics to paleobiology. This work will be performed in the Hadly lab at Stanford, which has a strong history of such genetic work and collaborations with museums. In particular, the Hadly lab is skilled at obtaining useable sequence data from ancient or otherwise poorly preserved, highly fragmented DNA samples. Thus we have confidence that where necessary, we can extract DNA from museum skins from AMNH (particularly from *Solenodon*, a critical taxon).

F. Funding

We have secured funding for this project. The Hadly lab has funds dedicated to this project. Mychajliw has applied for several independent grants as well, including $2,000 from the Society of Systematic Biologists. We have also receiving funds through supporting an undergraduate research assistant.

G. Appendix: List of All Specimens Requested for Project

Museum of Vertebrate Zoology, University of California Berkeley

Catalogue #	Species	Location	Preparation Type
MVZ Mammals 179735	Blarina brevicauda carolinensis	South Carolina	Whole organism (ethanol)
MVZ Mammals 183180	Blarina brevicauda talpoides	Massachusetts	Whole organism (ethanol)
MVZ Mammals 163012	Cryptotis mexicana mexicana	Mexico	Tissue (frozen)
MVZ Mammals 141669	Cryptotis nigrescens	Mexico	Whole organism (ethanol)
MVZ Mammals 192553	Suncus murinus	China	Tissue (frozen)
MVZ Mammals 185237	Crocidura attenuata	Vietnam	Tissue (ethanol)
MVZ Mammals 192172	Crocidura hutanis	Indonesia	Tissue (ethanol)
MVZ Mammals 196211	Crocidura grandiceps	Cameroon	Tissue (ethanol)
MVZ Mammals 117083	Myosorex varius varius	South Africa	Whole organism (ethanol)
MVZ Mammals 186403	Hylomys suillus ssp.	Vietnam	Tissue (frozen)
MVZ Mammals 181286	Scalopus aquaticus caryi	Nebraska	Whole organism (ethanol)
MVZ Mammals 225041	Neurotrichus gibbsii gibbsii	California	Tissue (frozen)
MVZ Mammals 191900	Hemiechinus auritus auritus	Iran	Tissue (frozen)
MVZ Mammals 179157	Diplomesodon pulchellum	Turkmenistan	Tissue (frozen)
MVZ Mammals 191907	Paraechinus hypomelas	Iran	Tissue (frozen)
MVZ Mammals 89513	Mogera insularis	China	Whole organism (ethanol)
MVZ Mammals 181063	Soriculus fumidus	Taiwan	Tissue (frozen)
MVZ Mammals 176815	Crocidura suaveolens	China	Tissue (frozen)
MVZ Mammals 180982	Anourosorex yamashinai	Taiwan	Tissue (frozen)
MVZ Mammals 172413	Erinaceus europaeus ssp.	Germany	Tissue (frozen)
MVZ Mammals 216901	Sorex oranatus oranatus	California	Tissue (frozen)

Museum of Southwestern Biology, University of New Mexico

Catalogue #	Species	Location	Preparation Type
MSB Mammals 95472	Neomys fodiens	Hungary	Organs (frozen)
MSB Mammals 94681	Neomys anomalus	Hungary	Organs (frozen)
MSB Mammals 151844	Blarina hylophaga	Kansas	Organs (ethanol)
MSB Mammals 95475	Sorex araneus	Hungary	Organs (frozen)
MSB Mammals 229822	Blarina brevicauda	New York	Organs (frozen)
MSB Mammals 48365	Talpa europaea	England	Organs (frozen)
MSB Mammals 47306	Scapanus latimanus anthonyi	Mexico	Tissue (frozen)
MSB Mammals 89528	Sorex monticolus	New Mexico	Organs (frozen)
MSB Mammals 99143	Notiosorex crawfordi	Texas	Organs (frozen)
MSB Mammals 45679	Urotrichus talpoides hondonis	Japan	Organs (frozen)
MSB Mammals 43387	Condylura cristata cristata	Massachusetts	Organs (frozen)
MSB Mammals 148576	Sorex caecutiens	Russia	Organs (frozen)
MSB Mammals 229151	Sorex palustris	Quebec	Tissue (frozen)

American Museum of Natural History

Catalogue #; Barcode	Species	Location	Preparation Type
M-114831	Nectogale elegans	Burma	Fluid, skin
M-187262	Surdisorex sp.	Kenya	Skin, skeleton, skull
M-274263; 101610	Blarinella griselda	Vietnam	Tissue, fluid
M-276730; 176199	Chimarrogale himalayica	China	Tissue, skeleton, skull, skin
M-49528	Sylvisorex megalura Irene	Zaire	Fluid
M-244222	Desmana moschata	Russia	Fluid
M-146627	Galemys pyrenaicus	France	Fluid
M-35331	Solenodon paradoxus	Dominican Republic	Fluid, skeleton, skull, skin
M-202361	Solenodon cubanus	Cuba/Zoo?	?
M-274154; 101500	Chodsigoa caovansunga	Vietnam	Tissue, fluid

To Be Determined

Source	Species	Location	Preparation Type
Personal Contact in Canary Islands	Crocidura canariensis	Canary Islands	Likely skin or skeleton

H. References

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