Identification of selected Pennsylvania mammals using hair

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Abstract: Hair is considered one of the synapomorphies (shared derived characters) of extant mammals. Keys and atlases based on hair are used for studies of food habits of predators, species identification of material recovered in the illegal trade of wildlife parts and products, determining diet changes in mammals, taxonomic and phylogenetic studies, and archaeology. These keys and atlases are used to identify hairs collected by noninvasive methods for censusing, such as hair traps and scat collection. In Pennsylvania, there are approximately 70 extant or extinct species of mammals. We examined guard hairs from mammalian species within the Commonwealth of Pennsylvania to (1) determine the synapomorphies of guard hairs from each family; (2) create a taxonomic tree and table for the mammals of Pennsylvania; and (3) create a tool for identifying mammal species that can be utilized with nonlethal sampling approaches.

Keywords: mammals, hair, atlas, dichotomous key

Mammals (class Mammalia) include 26 orders and over 5000 species. Hair is one of the synapomorphies (shared characteristics) that are unique to mammals, and all mammals have hair at some point in their development (University of Michigan Museum of Zoology 2013). Central cells of the hair follicle form the hair (Teerink 1991). The color of hair is derived from proteins known as melanins. Most hairs, other than human hairs, have bands of both eumelanin (which is dark) and pheomelanin (which is light) (University of Michigan Museum of Zoology 2013). The purpose of this research was to (1) determine the synapomorphies of guard hairs from each family; (2) create

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a taxonomic key and table for the mammals of Pennsylvania; and (3) create a nonlethal approach to sampling mammal species across their range.

MATERIALS AND METHODS

Various keys can be constructed, such as keys to scale patterns of guard hairs of mammals, or dichotomous key. Figure 1 is an example of a dichotomous key for the order Lagomorpha, showing how one may use physical characteristics to classify mammal hairs. This key was modeled following Debelica and Thies (2009). Hair samples were taken from specimens in the Penn State Department of Ecosystem Science and Management Bird and Mammal Collection. Each specimen has a Forest Resources (FR) number, beginning with the number 1. The American Society of Mammalogists' list of species of Pennsylvania mammals was followed to determine from which specimens to collect. At least ten dorsal guard hairs were collected from three specimens of each species. The entire hair was extracted to determine scale pattern throughout its length (Alaska Fur ID Project 2013).

A hot plate, a Pyrex glass measuring cup, and Knox gelatin were used to prepare the hairs for analysis of cuticular patterns. Approximately three drops of blue dye were added to improve visibility. The measuring cup was placed in a water bath on a Corning PC-400D hot plate, which was heated to approximately

Order Lagomorpha

Two species-*Sylvilagus floridanus* (Eastern Cottontail) and *Lepus americanus* (Snowshoe Hare). Both have cuticula imbricate, flattened and medulla continuous, nodose, symmetrical, occupies entire shaft.

1. Hair is white (winter pelage)..... *Lepus americanus* (Snowshoe Hare)
   Hair is not white..... 2
2. Largest MALDI-TOF peaks are between 1848 and 1956..... *Lepus americanus* (Snowshoe Hare) Largest MALDI-TOF peaks are not between 1848 and 1956..... 3
3. Largest MALDI-TOF peaks are between 1848 and 2064.. *Sylvilagus floridanus* (Eastern Cottontail)

Figure 1. Example from dichotomous key for the order Lagomorpha.

100°C. After 12–20 hours, it was heated to about 300–400°C to dissolve the gelatin. The slides were prepared by coating them with the gelatin solution.

Next, two or three hairs from each specimen were placed on the slide with the tip (apical) end attached, to make the hairs easy to remove. After the gelatin dried, the hairs were removed. Figures 2–4 illustrate the cuticular

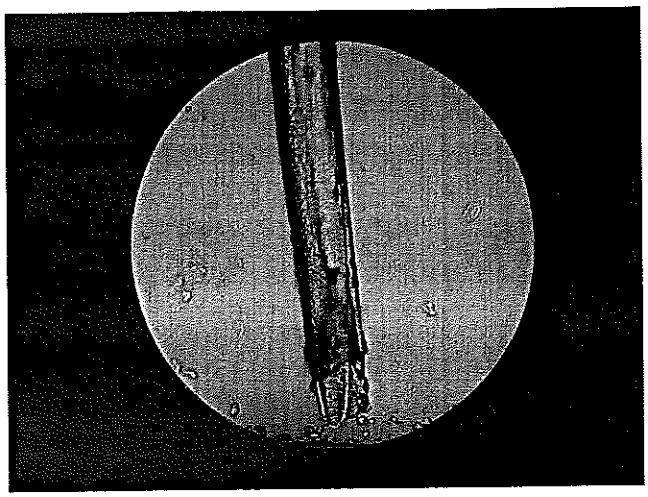


Figure 2. Virginia opossum (*D. virginiana*) cuticular tip scale pattern at 200X. See details in table 1.

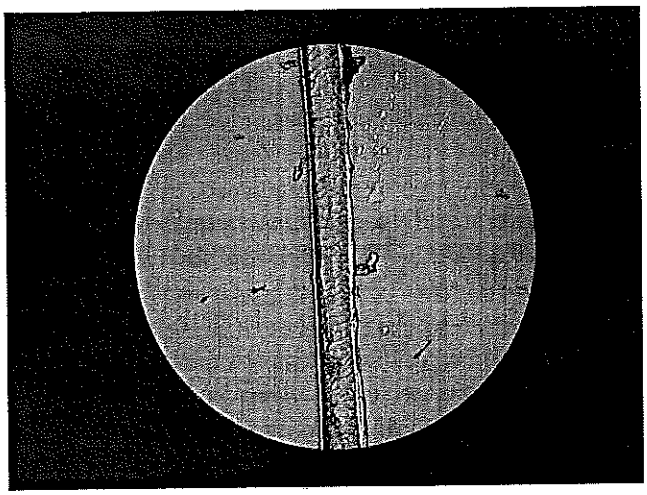


Figure 3. Virginia opossum (*D. virginiana*) cuticular middle shaft scale pattern at 200X. See details in table 1.

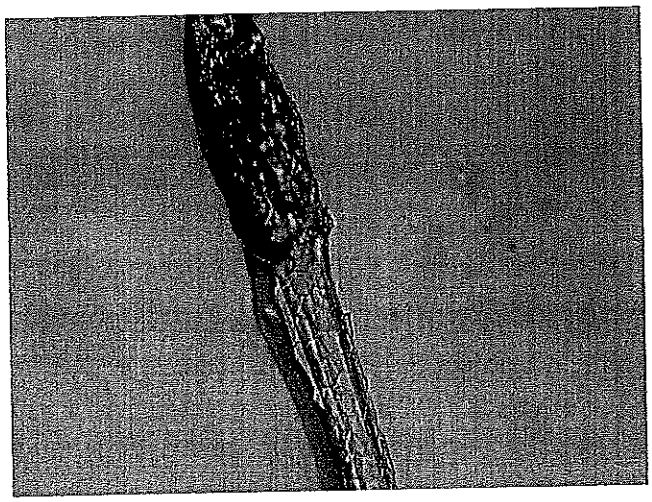


Figure 4. Virginia opossum (*D. virginiana*) cuticular root scale pattern at 200X. See details in table 1.

tip, middle shaft, and root scale pattern of a mounted hair from a Virginia opossum (*Didelphis*).

Medullar patterns were examined by using a glass dropper to add xylene on top of the hairs under a chemical hood. At least one hair from one specimen of each species was used, with the only exception being species with different summer and winter pelage, and albino specimens; in these cases, a second hair and slide were used. Larger hairs were cut in half cross-sectionally with a razor blade to enhance absorption of the xylene. After the xylene dried, Duco cement was added to each end of each hair, to secure the hair on the slide. For species with darkly pigmented fur, the hairs were placed in hydrogen peroxide in a glass Pyrex dish, to improve visualization of the medulla (Sahajpal and Goyal 2009); however, the hydrogen peroxide appears to have worked very little, or not at all. Figure 5 exhibits the medullar pattern of a mounted Virginia opossum (*D. virginiana*) hair. Table 1 has information pertaining to the hair samples used in figures 2–5.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, performed at the Penn State College of Medicine in Hershey, was also used. This is also known as the SIAM method (Species Identification of Animals with MALDI-TOF mass spectrometry). It works by pretreating hairs by cooking them in a chemically reducing solvent. A trypsin solution cuts the keratin chains into peptides. These peptides are of different lengths and molecular weights. Mass spectrometry arranges them

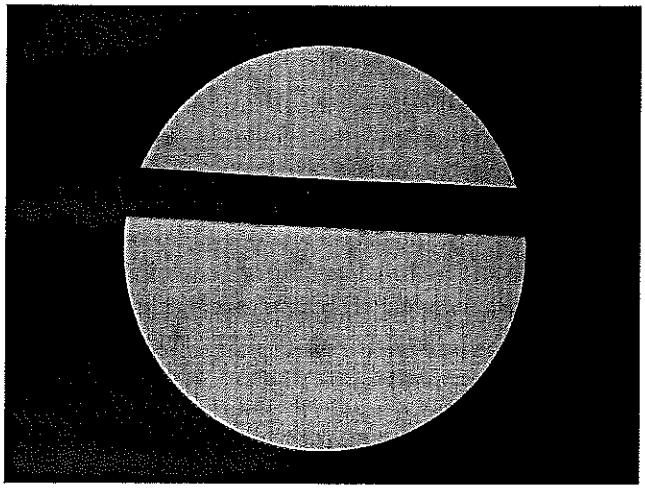


Figure 5. Virginia opossum (*D. virginiana*) Medullar Pattern at 200X. See details in table 1.

Table 1. Information pertaining to hair samples used in figures 2–5.

Virginia Opossum—*Didelphis virginiana*
Cuticula imbricate, crenate
Medulla continuous, nodose, occupies more than 1/2 of shaft
Salient features: long, white, gray, or sometimes yellowish
Average total length: 5.016 cm
Average width at tip: 0.533 µm
Average width at middle shaft: 2.433 µm
Average width at root: 3 µm

according to their molecular weights, which form a peptide mass fingerprint (pmf) that is distinctive to each species. Species can be identified, and relationships determined, by this spectrum pattern (Hollemeyer et al. 2002). The SIAM method of hair identification is currently accepted in the European Union for commercial hair analysis, and by German customs. A weigh boat was placed on an OHAUS Explorer scale and zeroed. The hairs were weighed to the nearest tenth of a milligram. The hairs were then transferred to 0.65 mL centrifuge tubes with tweezers. After each transfer, the tweezers were cleaned with 95% EtOH and 2% bleach and then rinsed in reverse osmosis water. The centrifuge tubes were labeled 1–53, corresponding to each sample.

All slides were labeled and then viewed with 200× magnification with a Leica DMR light microscope. The microscopic images provided by each cast were photographed with a Nikon COOLPIX 5000 digital camera on the microscope and recorded on a spreadsheet and in a notebook. Often these studies are conducted using a scanning electron microscope (SEM), transmission electron microscope, or both (Clement et al. 1980, Sessions et al. 2009). A recent technique is to view the cuticle with a light microscope first, and then with a scanning electron microscope (Clement et al. 1980). Light microscopy has been used in earlier studies, such as Hausman (1920) and Sessions et al. (2009), and also by Sahajpal et al. (2007). The data were recorded on two Excel spreadsheets. The columns listed the common name of the species, the (FR number, cuticula type, comments, medulla type, total length (cm), salient features (features that can be seen with the naked eye, such as color and length), and two extra measurements for bat species: scale index (SI) and width index (WI). Width (μm), number of scales across width (scale count), and photo number were recorded for each of the three parts along the length of each hair (tip, middle shaft, and root). One to three specimens were used for each species, and as many as three hairs for each species. Measurements across the row of the spreadsheet corresponded to each hair. The FR column has an asterisk (*) if the number of the specimen was missing, or if the specimen from which the hair came was not recorded. Sometimes the scale pattern would change along the length of the hair, requiring a fourth photo, and sometimes a fourth width measurement. A quantitative key to the hair of mammals from Pennsylvania was constructed.

RESULTS AND DISCUSSION

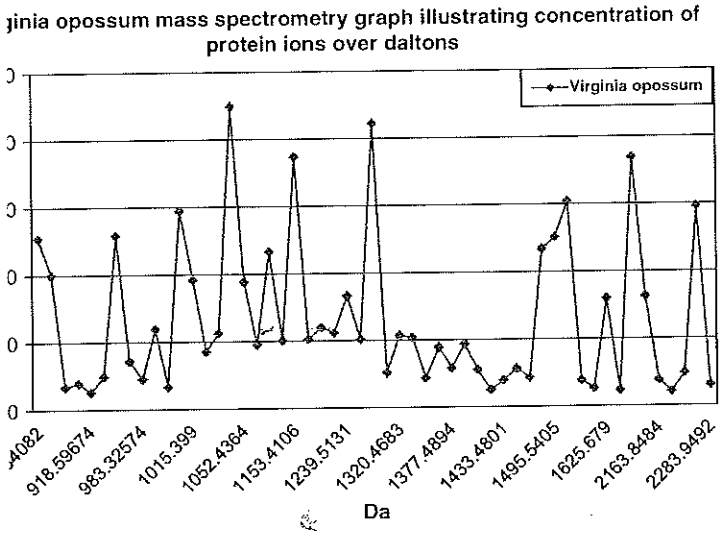
The MALDI-TOF mass spectrometry was the most accurate and reliable method of distinguishing mammals by their hair. There was no missing information, unlike for the medullar and cuticular patterns. It is not subjective (i.e., one cuticular or medullar type might be given a different name by different scientists, or what one scientist considers two different cuticular types may be classified as two different subtypes of the same type). Furthermore, this method was effective irrespective of whether the samples were derived from juveniles or adults, summer vs. winter pelage, or domestic vs. wild mammals. Some species had a medullar pattern that did not appear under the microscope or in the photographs, because it was too dark. MALDI-TOF gives objective, numerical results, as opposed to using a subjective classification system (there is also more than one way to categorize cuticular and medullar patterns, by different scientists and authors). All photographs of each hair are available in Nickoloff (2013).

The average widths and lengths were predictable, for the most part, with some exceptions, such as the southern flying squirrel (*Glaucomys volans*) having a wider tip, middle shaft, and root than the northern flying squirrel (*Gl. sabrinus*). This, however, could be explained by human error in measurement, labeling, or calculation, or as a result of seasonal pelage length (i.e., shorter in summer and longer in winter). With few exceptions, when certain species' hairs appeared to be longer and wider than those of other species without the microscope, that was actually true, according to the measurements.

Although the widths of the hairs differed, there was a noticeable pattern: the tip usually had the smallest width, the middle shaft had the largest width, and the width of the root was in between. Most species seemed to have a cuticula that was either imbricate, crenate, or imbricate, flattened. All shrew and mole species in this key and atlas had an imbricate, elongate cuticula. Similarly, all bat species had a coronal cuticula, but the specific type of coronal was not able to be determined with the light microscope. The porcupine hairs were measured and photographed under 50× magnification, because otherwise the entire hair would not be visible (they appeared very large under the microscope). While a flattened cuticula is defined as having one or two scales, hairs of some species with a flattened cuticula had more than two scales. Albino hair had no effect on medullar type, but the medulla was more easily visible under the microscope and in photos. Since hair color is mainly due to pigment granules (Hausman 1924), albino hairs would be no different in structure from typical mammal hairs. It would be difficult to distinguish the bat species based solely on scale index and width index, since the scale index measurements were not very different between species, and all had the same width index, which was 2, except the Keen's myotis (*Myotis keenii*), which had a width index of 3 (its hair is noticeable, microscopically, as wider than hairs of other bats). The cuticular and medullar types of all species could probably be determined with a scanning electron microscope, which was not available. The house mouse (*Mus musculus*) and Norway rat (*Rattus norvegicus*) hairs both have a distinct cuticula (imbricate, ovate and imbricate, acuminate, respectively).

According to a study by Hollemeyer et al. (2002), some MALDI-TOF mass spectrometry peaks are found in at least 80% of the mammal species tested. Those that are not are referred to as semispecific peaks, or SEMPs (which can be further divided into unique species-specific peaks, or USSPs, and more frequent peaks occurring in several species, or MFGs). These can be used to identify species relationships (2002), with the largest peaks being the most useful. If all peaks are the same, the two or more specimens are the same species. Figure 6 is an example of a mass spectrometry graph showing guard hair protein composition for the Virginia opossum (*D. virginiana*).

t mammal specimens have been classified by their dental pulp with OF mass spectrometry, and this method can be used along with DNA g, especially with ancient specimens, or for forensic purposes (Tran). Three of the four shrew species, the smoky shrew (*Sorex fumeus*), shrew (*S. fontinalis*), and northern short-tailed shrew (*Blarina* ı), had similar MALDI-TOF peaks at 1504, 1503, and 1504, respectively, ːak from one was very different: the masked shrew (*S. cinereus*), pos- gh peak at 805. As one might expect, the two moles included above, the d mole (*Parascalops breweri*) and star-nosed mole (*Condylura cristata*), eak at 1263, and the eastern cottontail (*Sylvilagus floridanus*) and snow- (*Lepus americanus*) both have a peak at 1848 (Table 3-1 in Nickoloff : two introduced Muridae species, the house mouse (*M. musculus*) and ıt (*R. norvegicus*), have a peak in common at 1164, while two of the three ːcies, the white-footed mouse (*Peromyscus leucopus*) and deer mouse ılatus), share a peak at 805. The Allegheny woodrat's (*Neotoma magis-* peak is similar to that of the white-footed mouse (*P. leucopus*) at 2070. domestic dog (*Canis familiaris*) does not share any peaks at the exact ıber as either of the two fox species, the red (*Vulpes vulpes*) and gray ːcyon *cinereoargenteus*), they both have a peak at 1011; the domestic dog ıris), surprisingly, shares a peak at 1038 with the raccoon (*Procyon lotor*).



Example (Virginia opossum, *D. virginiana*) from Pennsylvania Mammal Species Mass etry of Guard Hair Protein Composition.

These carnivores were chosen for this SEMP comparison mainly because of these unusual results. While the white-tailed deer (*Odocoileus virginianus*) has peaks at 1109 and 1505, the moose (*Alces alces*) has two peaks at 1107 and 1503. The peaks of the bats of Pennsylvania are not very far apart: 1435, 1459, and 1453 for the red bat (*Lasiurus borealis*), tri-colored bat (*Perimyotis subflavus*), and big brown bat (*Eptesicus fuscus*), respectively. The red bat (*L. borealis*) and tri-colored bat (*Per. subflavus*) also have similar peaks at 1546 and 1548, respectively.

Some species can be separated by gross features, such as hair color or pattern. When a species has different summer and winter pelage, the species must appear more than once in the key. MALDI-TOF mass spectrometry may be the only way to distinguish some species in a dichotomous key, because the cuticular and medullar types may be the same for all species in an order, and they may also have the same hair color or pattern. This is also true for domestic animals and humans, because their hair can vary greatly.

Some disadvantages of MALDI-TOF mass spectrometry are that it can be time-consuming and expensive; if a species can be separated out by gross features, then mass spectrometry is not necessary. Also, some results may not seem accurate, such as the shared semispecific peaks of the domestic dog (*C. familiaris*) and raccoon (*Pr. lotor*), or the masked shrew (*S. cinereus*) not having a shared peak with the others shrew species. Therefore, other methods must be used. In conclusion, the three methods (cuticular patterns, medullar patterns, and MALDI-TOF mass spectrometry) should be used together. Future research should include both a larger sample size and statistical analysis.

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