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Discrimination between *Peromyscus leucopus noveboracensis* and *Peromyscus maniculatus nubiterrae* in the field

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Syntopic *Peromyscus leucopus noveboracensis* (Fischer, 1829) and *P. maniculatus nubiterrae* (Rhoads, 1896) exhibit considerable overlap in external morphologies in the Appalachian mountains of the Eastern United States, making field identification of live individuals questionable in some instances. We compared two techniques for correctly identifying these live individuals in the field: the tail:body ratio and weight criterion, and discriminant function analysis of external morphological characters. Electrophoresis of salivary amylase was used to confirm species identity. The tail:body ratio and weight criterion was a poor character combination for discriminating between species, with 36% of new (unmarked) individuals misclassified. Models generated from discriminant function analysis resulted in up to 92% correct classification to species of live individuals. For *any* individual *Peromyscus* (Gloger, 1841) captured, four quantitative characters (tail length, body length, ear length, and weight) were the most useful in discrimination between species. While classification equations provided improved species identification, they still resulted in a high degree of error. Only electrophoresis of salivary amylase provided unambiguous species identification in the field, and we recommend the use of this technique.

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Introduction

In any ecological investigation, it is critical to positively identify species. The white-footed mouse, *Peromyscus leucopus noveboracensis* (Fischer, 1829), and the cloudland deer mouse, *Peromyscus maniculatus nubiterrae* (Rhoads, 1896) coexist in the deciduous forests of the central Appalachian Mountains of the Eastern United States (Wolff 1985, Bruseo and Barry 1995). These species exhibit substantial overlap in both niche requirements and morphology (Wolff 1985). Such overlap has prompted numerous investigations into the presence of resource partitioning and its role in mediating interspecific competition between these mice. Assessment of such ecological mechanisms is highly dependent on correct species identification. Misidentification of one or a few individuals can lead to commission of Type I or

Type II errors, especially when statistical results approach significance (eg α = 0.05). For example, tests for differences between activity times of *P. leucopus* and *P. maniculatus* indicated that a hypothetical misidentification of one individual out of 64 changed log likelihood test results from significant (p = 0.034) to non-significant (p = 0.124; unpublished data).

Where they are sympatric, similarity in external morphology of these two *Peromyscus* Gloger, 1841 species makes field identification questionable in some instances. In New England, *Peromyscus leucopus noveboracensis* typically have a chestnut colored pelage with a well-defined mid-dorsal stripe and uniformly colored tail, while *P. maniculatus gracilis* (Le Conte, 1855) exhibit dark brown to greyish pelage and a sharply bicolored tail with a 'pencil' tip (Choate 1973). The Choate (qualitative) criterion has also been used to distinguish between *P. l. noveboracensis* and *P. maniculatus nubiterrae* in the Appalachians. A number of investigators (Wolff *et al.* 1983, Wolff 1985, Harney and Dueser 1987) have extensively examined competitive interactions between these two species, but do not indicate their criteria for species identification. Other studies (Barry *et al.* 1990, Bruseo and Barry 1995) have relied heavily on the criterion of Choate (1973) in distinguishing between these two *Peromyscus* species. While the features described by Choate (1973) are considered 'typical', they are subjective, exhibit much variability, and may be unreliable when distinguishing between these two species.

Positive species identification of *P. l. noveboracensis* and *P. m. nubiterrae* can be achieved using biochemical markers. Polyacrylamide gel electrophoresis (PAGE) of salivary amylase offers a molecular method for positive species identification, and has been used to distinguish between *P. leucopus* and *P. maniculatus* (Aquadro and Patton 1980, Feldhamer *et al.* 1983, Parren and Capen 1985, Palas *et al.* 1992). While this technique provides unquestionable species identification, its use is limited in field experiments. The procedure can be relatively time and labor intensive, taking from 9 to 12 hours in the laboratory to prepare, run, and develop gels. More importantly, it does not provide immediate species identification, which may be required in investigations where one species is selectively removed.

Morphometric analysis is also useful in distinguishing between syntopic *Peromyscus*. Feldhamer *et al.* (1983) examined standard body measurements of *P. leucopus* and *P. maniculatus* in the Appalachian mountains of western Maryland to determine objective characteristics for identification of these species. A tail length / head and body length (tail:body) ratio was calculated from measurements taken on snap-trapped (dead) adult (males and non-pregnant females) and subadult specimens. Species verification was made using electrophoresis of salivary amylase. The authors found that tail:body ratios provided correct species identity 76.7% of the time. In cases where ratios overlapped, weight increased correct identification to 93.2%. However, head and body length measurements may be unreliable in the field, particularly when dealing with live, active animals. Using the same criterion (tail:body ratio and weight), Sternburg and Feldhamer (1997) successfully distinguished between *P. leucopus leucopus* (Rafinesque, 1818) and *P. maniculatus*

bairdii (Wagner, 1845) in southern Illinois up to 98.6% of the time. Rich *et al.* (1996) used quantitative cranial characteristics to distinguish between *P. leucopus* and *P. maniculatus* in northeastern North America. The authors developed a 12-variable discriminant equation that correctly classified 100% of the specimens. While all of these investigations provide successful species identification, they are based on measurements obtained from *dead* animals, and may not be applicable to field studies of behavioral ecology where individuals are followed over time.

The purpose of our investigation was to identify a field technique that would accurately distinguish between live *Peromyscus leucopus* and *P. maniculatus*. Because our study site was close (ca 48 km) to the field site of Feldhamer *et al.* (1983) in western Maryland, we first tested the reliability of Feldhamer's criterion (tail:body ratio and weight) on *live* mice. Body length measurements taken on live animals are expected to be less accurate than those taken on dead animals because of the constraints of immobilizing live animals for precise body measurements. Therefore, we hypothesized that tail:body ratio and weight may be unreliable characters for identification of animals in the field. Second, using discriminant function analysis (DFA), we identified the minimum subset of ten (five quantitative and five qualitative) morphological characters collected that would yield the most accurate species identification.

Material and methods

Data Collection

This study was conducted as part of an investigation of interspecific competition at the Powdermill Nature Reserve, Westmoreland County, PA. From 15 May to 29 October 1995, mice were trapped on four 7 × 7 live-trapping grids (trap interval of 15 m). For each *Peromyscus* captured, sex, age based on pelage characters (Osgood 1909) and weight, and reproductive status were recorded. Three quantitative measures (weight to the nearest 0.5 g, tail length and body length) were obtained for every individual. In addition, ear length and hind foot length were obtained for most individuals. Quantitative measures were taken to the nearest mm using a clear, flexible plastic ruler. Body length was obtained by molding the ruler from the tip of the nose to the base of the tail of each mouse. Body measurements taken on individuals captured in the field, and later anesthetized, were different, with field measurements consistently overestimating body length. While this technique differed from the standard method of obtaining a body measurment and may have influenced results, we opted to use this technique because it yielded a consistent measurement with minimal stress to the animal. Five qualitative measures were recorded for every individual. These consisted of pelage by age (0 = gray, 1 = brown), adult brownness (0 = chestnut brown, 1 = not chestnut brown), tail bicoloredness (0 = uniform, 1 = bicolored), hairiness of tail (0 = not hairy, 1 = hairy), and paintbrush (pencil) tip to tail (0 = absent, 1 = present). In instances where characters could be considered continuous (eg, tail bicoloration), any indication of that character (weak to strong) was scored as presence of that character. All measurements were taken by the senior author. Animals were marked by toe-clipping, and released at the site of capture. Prior to animal release, a sample of salivary amylase was obtained from each individual by rinsing the mouth of the animal with approximately 1 ml distilled water, and collecting the wash in a 1.5 ml Eppendorf tube. Each tube was labeled with the animal's identification number and date of collection. Samples were immediately placed on wet ice, and later frozen upon return from the field.

In addition to live trapping, fifteen dead specimens were collected from one night of snap trapping at a site 3.2 km from our study site. Measurements from these animals were used to test the reliability

of the Feldhamer criterion in classifying dead *Peromyscus*. Species identification was achieved through electrophoresis of salivary amylase. Since our objective was to identify characters that would positively classify live animals, none of the snap-trapped animals were used in discriminant function analysis.

Electrophoresis

Amylase samples were prepared for electrophoresis by pipetting 50 μl raw sample, 50 μl Tris-Cl sample buffer (pH = 6.8), and 25 μl Bromophenol blue solution into a 0.5 ml micro-centrifuge tube. Electrophoresis of salivary amylase was carried out using 0.75 mm × 14 cm × 14 cm non-denaturing discontinuous polyacrylamide gels. Gels were prepared based on the following modification of the protocol described by Smith (1989). Separating gels were prepared by combining 5 ml of a 30% acrylamide / 0.8% bis-acrylamide solution, 7.5 ml of Tris-Cl (pH = 8.8), 17.5 ml dH2O, 1.5 ml ammonium persulfate, and 50 μl TEMED. Separating gels were poured and allowed to polymerize 20–30 minutes at ambient temperature. Stacking gels were prepared by combining 1.95 ml 30% acrylamide / 0.8% bis-acrylamide solution, 3.75 ml Tris-Cl (pH 6.8), 9.15 ml dH2O, 200 μl ammonium persulfate and 25 μl TEMED. This solution was poured over the separating gel, and a 20-well comb was inserted between the glass plates. Stacking gels were allowed to polymerize from 15–20 minutes. After polymerization, the comb was removed, and the wells were rinsed with 1X Tris-glycine electrophoresis buffer (pH = 8.3). The resulting gel was clamped to the upper chamber of a Vertical Gel Electrophoresis System (Bethesda Research Laboratories, Gaithersburg, MD). The upper and lower chambers of the apparatus were filled with a total volume of 1500 ml 1X Tris-glycine electrophoresis buffer (pH = 8.3) ensuring that buffer extended 1–2 cm above the top of the wells.

Fifteen microliters of prepared sample were placed into each well, and gels were electrophoresed at 25–30 mA for 5–6 hours under cold (5°C) conditions. When the tracking dye reached the bottom of the separating gel, power was turned off. Gels were marked to orient well location, and placed in a solution of 1% starch: 2% Tris (pH = 7.4): 1.5% CaCl for 6–12 hours at 5°C. Gels were then rinsed with dH_2O, stained for 1–3 minutes in a dilute (8 dH_2O : 1 concentrated solution) I_2KI solution (10 g potassium iodide, 2 g iodine crystals, 500 ml dH_2O), and rinsed with dH_2O. Staining produced clear bands on a blue-black background. Gels were fixed with a solution of methanol:distilled water:glacial acetic acid (5:5:1) for 2–4 minutes, then rinsed with distilled water. Bands were scored by measuring migration distance (mm) from the bottom of the wells.

Constructing Classification Models

Different combinations of quantitative and qualitative morphological characters were subjected to discriminant function analysis (DFA) to determine the minimal number of characters required for the most accurate species classification. Many sources warn against biases that may occur when using stepwise discriminant function analysis (Tabachnick and Fidell 1983, Wilkinson 1992, Manly 1994). Therefore, we subjected multiple combinations of variables to DFA, nine of which are presented here. Over 30 different combinations of variables were tested, beginning with inclusion of all ten variables, then proceeding with the systematic elimination of variables until the lowest percent misidentifications using the fewest variables were observed. Additionally, combinations of variables commonly used to identify species (eg, Feldhamer criterion of tail:body ratio and weight) were tested. Prior to inclusion in the analyses, data were tested for linearity, homogeneity of variance/covariance matrices, multicollinearity/singularity, and univariate normality (Tabachnick and Fidell 1983), and met these assumptions. Discriminant function analysis is sensitive to outliers (Tabachnick and Fidell 1983). However, we included individuals considered to be outliers in the analyses to account for the natural variation in morphological characters. This improved the utility of the models.

The complete database (All Animals: n = 155) and four subsets (Adults: n = 82; Adult / Subadult: n = 144; All Males: n = 79; All Females: n =76) were analyzed to construct the models that would best identify individuals to species. The Adult dataset included only adult animals for which all ten morphological characters were recorded, and excluded animals that were infested with botfly larvae, pregnant, or had incomplete tails. Such animals could have introduced undue bias to the models. For example, higher weights of pregnant females may increase the importance of that variable in DFA when in fact weights may not contribute to discrimination between species. Because the model

resulting from analysis of the Adult dataset was restrictive in its application to field situations and our goal was to develop a technique that could be applied to *any* individual, additional datasets were analyzed that included all individuals captured, regardless of age or reproductive condition. The All Animal dataset included every animal captured during the trapping period. In the Adult / Subadult dataset, juveniles were excluded from analysis. To determine if there were differences among morphological characters of the sexes, All Males and All Females were analyzed separately. In constructing all models, only one record per animal was used (ie, a mouse initially captured as a juvenile and later captured as an adult was only included in the analysis as an adult).

Models were generated by subjecting the five datasets to discriminant function analysis using SYSTAT for the Macintosh, Version 5.2 (Wilkinson 1992). The analyses provided percent correct classification of individuals to species. A Wilks' lambda value was also generated by DFA, which allowed evaluation of the discriminatory power of the models. Wilks' lambda values range from 0 (perfect discriminatory power) to 1 (no discriminatory power; Statsoft 1994). For each dataset, nine trials (different combinations of morphological characters) were subjected to discriminant function analysis. The trial that produced the lowest percent misidentification using the fewest number of characters was considered the best fit model for the dataset. Outputs generated from DFA provided classification coefficients for characters included in the best fit model. A single classification equation was generated for each model by subtracting the classification coefficients for the *P. maniculatus* equation from the coefficients in the *P. leucopus* equation. Species identity could be determined in the field by entering measurements for the corresponding variables into the single equation and solving it. Individuals with positive scores were identified as *P. leucopus*, while those with negative scores were classified as *P. maniculatus*.

The best fit models (Appendix 1) were field-tested by trapping two of the four grids from 3–6 October 1996. Only new (unmarked) mice (n = 32) were tested, providing an independent sample with which we assessed the accuracy of the models. All morphological characters and a salivary amylase sample were taken for each individual captured. Each model was tested by entering the specific variables into and solving the classification equations. Species identity was assigned using classification equations and confirmed with electrophoresis of salivary amylase.

Results

Electrophoresis

Polyacrylamide gel electrophoresis of salivary amylase revealed distinct electromorphs for each of the two *Peromyscus* species. No heterozygotes were observed at the salivary amylase locus for either population. Either a fast-migrating band and a slow-migrating band was observed, which corresponded to the *Amy-1*^{100} (fast) band of *P. leucopus* and *Amy-1*^{76} (slow) band of *P. maniculatus* reported by Aquadro and Patton (1980).

Model Construction

When the Feldhamer *et al.* (1983) criterion was used to determine species identity of live mice, 30 out of 79 *P. leucopus* (19 of 66 adults, 7 of 9 subadults, 4 of 4 juveniles) were misidentified, and three adults out of 75 *P. maniculatus* were misidentified, for an overall misidentification rate of 21% (33 out of 154 total animals). When only adults were classified using the Feldhamer criterion, 19 out of 65 *P. leucopus* and three out of 56 *P. maniculatus* were misidentified (18% total misidentification rate). No significant differences were observed between the misidentification rates of the sexes. Use of tail:body ratio and weight in classifying

Table 1. Evaluation of datasets subjected to discriminant function analysis for the best fit models. Note: Bold values indicate the best fit model for each dataset; *n* – number of individuals included in analysis, % – percent misidentification, t – tail lenght (mm), b – body length (mm), e – ear lenght (mm), hf – hind foot length (mm), w – weight (g), pel – pelage color, pb – paintbrush tip to tail, bi – bicoloredness of tail, hair – hairiness of tail, r – tail length:body length ratio, * – Choate criterion, ** – Feldhamer *et al.* criterion, *P.l.* – *Peromyscus leucopus*, *P.m.* – *Peromyscus maniculatus*.

Trials (characters used in DFA)	Adult dataset (*n* = 82)					All animals dataset (*n* = 155)					Adult / Subadult dataset (*n* = 144)					All males dataset (*n* = 79)					All females dataset (*n* = 75)				
	Wilks'	*P.l.*		*P.m.*		Wilks'	*P.l.*		*P.m.*		Wilks'	*P.l.*		*P.m.*		Wilks'	*P.l.*		*P.m.*		Wilks'	*P.l.*		*P.m.*	
	λ	*n*	%	*n*	%	λ	*n*	%	*n*	%	λ	*n*	%	*n*	%	λ	*n*	%	*n*	%	λ	*n*	%	*n*	%
t,b,e,hf,w,pel, pb,bi,hair	0.22	41	2	41	0	0.31	49	6	42	3	0.30	46	4	39	3	0.21	28	0	21	0	0.26	20	5	20	0
t,b,e,w,pel,pb, bi,hair	0.22	41	2	41	0	0.31	76	4	67	3	0.30	71	3	63	3	0.21	43	0	32	3	0.31	33	9	36	6
t,b,e,hf,w	0.25	41	0	41	7	0.37	49	6	42	5	0.36	46	4	40	3	0.27	28	0	21	10	0.31	20	15	20	0
t,b,e,w	0.26	41	0	41	5	**0.39**	**76**	**4**	**69**	**4**	0.38	71	4	63	5	0.31	43	5	32	6	0.37	33	6	36	3
* pel, pb, bi, hair	0.59	41	3	41	12	0.65	79	23	75	20	0.65	74	23	67	18	0.64	43	21	35	20	0.60	36	22	40	20
** r,w	0.38	41	12	41	15	0.62	78	15	77	14	0.56	73	19	68	15	0.44	43	12	36	17	0.58	35	26	40	13
t,w,e	**0.28**	**41**	**0**	**41**	**0**	0.56	76	8	69	6	0.38	71	6	63	5	0.31	43	5	32	6	**0.62**	**33**	**3**	**36**	**3**
t,b,e,bi,w	0.22	41	3	41	0	0.32	76	8	69	3	**0.32**	**71**	**6**	**63**	**3**	0.22	43	0	32	0	0.34	33	9	36	3
t,e,bi,w	0.27	41	5	41	2	0.32	76	8	69	4	0.32	71	6	63	3	**0.22**	**43**	**0**	**32**	**0**	0.35	33	9	36	3

fifteen snap-trapped (dead) animals collected near the study site resulted in no misidentified animals.

Similar results were found when subjecting the Feldhamer *et al.* (1983) criterion to discriminant function analysis (see Table 1). For all datasets, the variables tail:body ratio and weight resulted in high misclassification rates (12.5–17 % overall misclassification). Qualitative characters (pelage by age, brownness, bicolored tail, paintbrush tip, and hairiness of tail) alone were also poor predictors of species identification, with a 15.6–21.4% overall misclassification rate. Both the Feldhamer *et al.* (1983) criterion and the Choate (1973) criterion (qualitative characters) produced the highest Wilks' lambda values, indicating relatively poor discriminating power of these character combinations. The error rate for both of these character sets indicated a need for different criterion in species discrimination of live animals.

Each of the five datasets tested produced a different best fit model. Analysis of the Adult dataset produced a best fit model (Appendix 1) in which tail length, weight, and ear length provided 100% correct species classification (see Table 1). While this combination produced a Wilks' lambda value slightly higher than other combinations, it provided correct classification in all cases using the minimum number (three) of characters (Fig. 1a). This combination of characters also provided the best fit model for the All Female dataset. The best fit model for the All Animals dataset (Appendix 1) included four quantitative characters (tail length, body length, ear length and weight), resulting in six out of 154 (3.9%) individuals misclassified (Fig. 1b). Bicoloration of the tail, in combination with the four quantitative characters, was important in discriminating between individuals in the Adult / Subadult and All Males datasets (see Appendix 1).

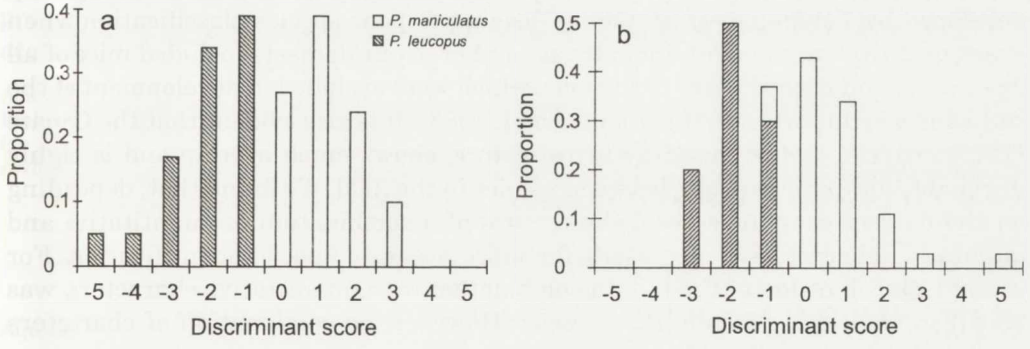

Fig. 1. Discriminant scores of individuals subjected to discriminant function analysis: (a) Adult Dataset (n = 82) using tail length, ear length and weight; (b) All Animals Dataset (n = 154) using tail length, body length, ear length and weight. Discriminant scores are standardized across all variables for each individual, and indicate group membership.

Field Testing of Models

Subsequent field testing of the best fit models using 32 new individuals (24 *P. leucopus* and 8 *P. maniculatus*) indicated a number of misidentifications. The best fit model for the All Males dataset resulted in the lowest percentage of misclassified individuals (1 out of 13, or 7.7%). The highest misidentification rate was observed for the best fit model of the All Female dataset (4 out of 19 individuals, or 21%). Three out of 32 individuals (9.4%) were misidentified when the best fit model for the All Animals dataset was tested. For all best fit models, from 8 to 21% of new individuals were misidentified. The Feldhamer criterion for identification of new individuals resulted in a 36% misidentification rate (11 out of 31 individuals), while use of the Choate criterion yielded a 38% misidientification rate (12 out of 32 individuals).

Discussion

It is clear from electrophoretic evidence and discriminant function analysis that no combination of characters can be used to unambiguously identify live *Peromyscus* in the field. The Feldhamer criterion is unreliable for prediction of species identity using live mice in the field. An unacceptably high (36%) proportion of unmarked animals were misidentified using this criterion. This misidentification rate was higher than that for individuals used in constructing classification models (18–21%), and may be the result of smaller sample sizes used in the field test. These characters are reliable when classifying dead animals, as indicated by the correct classification of the fifteen snap-trapped individuals. The disparity in classifying live versus dead animals using ratio and weight may be attributed to inaccurate body measurements obtained on live, active animals. Differences in measurements from our study changed the distribution of tail:body ratios originally developed by Feldhamer *et al.* (1983), leading to poor species classification when examining live animals. Additionally, a number of our datasets included mice of all age classes and reproductive conditions, which were excluded in development of the ratio and weight criterion (Feldhamer *et al.* 1983). It is also evident that the Choate (1973) criterion, widely used by investigators, shows much overlap and is highly unreliable for distinguishing between species in the field. We found that, depending on the dataset examined (see Table 1), use of a combination of quantitative and qualitative characters is necessary for more accurate species identification. For example, bicoloration of the tail, in combination with quantitative characters, was useful in identifying female *Peromyscus*. However, no combination of characters produced definitive identification.

Our analyses indicated that up to five morphological characters provided good, but not perfect, discrimination regardless of the dataset examined. Tail length, ear length, and weight appeared in all five best fit models. These measurements can be quickly obtained on live animals in the field. When animals other than adults were

included in DFA, body length and bicoloredness of the tail also became important characters in discriminating between species (males and adult / subadult datasets). It was surprising that body length appeared in two of the five best fit models since we anticipated that this measurement would be inaccurate in the field. Body length became important when analyses included all age classes and reproductive conditions. While our models were an improvement over currently used identification techniques, they did not provide unquestionable species identity.

Our models produced a number of misclassifications when tested on new individuals, which translated to an unacceptably high percentage of misidentifications. Of the individuals tested, one *P. leucopus* was misclassified as a *P. maniculatus* in every model. This adult female fit all the character traits considered 'typical' for *P. maniculatus*, but salivary amylase indicated this female was in fact a *P. leucopus*. This individual demonstrates the potential problem with using untested 'rules of thumb' for classifying individuals, and the need for a more rigorous approach to species identity, particularly where they show overlap in external morphology. The models presented do not provide an infallible technique for classification of live *Peromyscus* in the field. These models are also restrictive in their application due to variability in morphological characters of *Peromyscus* over large geographic areas. The only unquestionable method of field identification of these two species is through electrophoresis of salivary amylase.

Correct identification of individuals to species is necessary to maintain integrity in conclusions drawn regarding ecological processes. This issue has been largely neglected or dismissed by many investigators studying interactions (such as competition) between these species. A few misidentified individuals may not be critical in cases where investigations are broadly based (eg, population density estimates), or where large populations are being studied. When the focus of species interactions is at the level of the individual (eg, differences in activity times or home range), correct species identification becomes critical. Once individuals are properly identified, the true interactions that occur within or among species can be accurately assessed.

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Appendix 1. Classification equations (C) for the best fit models using five different datasets in discriminant function analysis.

Best fit model for Adult dataset
C Adults = 38.537 + (–0.465)(tail length) + 0.979(weight) + (–1.283)(ear length)
Best fit model for All Animals dataset
C All Animals = 25.524 + (–0.208)(tail length) + 0.046(body length) + (–1.486)(ear length) + 0.499 (weight)
Best fit model for Adults and Subadults
C Adults and Subadults= –40.933 – 0.566 (tail length) + 0.118 (body length) – 1.408 (ear length) – 3.365 (bicoloredness) + 1.145 (weight)
Best fit model for Males only
C Males = –37.909 – 0.351(tail length) – 2.402 (ear length) – 5.584 (bicoloredness) + 1.725 (weight)
Best fit model for Females only
C Females = –95.17 – 1.408 (tail length) – 2.313 (ear length) + 1.313 (weight)