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Comparison of Morphological Versus Molecular Characters for Discriminating Between Sympatric Meadow and Prairie Voles

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ABSTRACT.—Prairie (*Microtus ochrogaster*) and meadow (*M. pennsylvanicus*) voles are morphologically very similar species of rodents that are often found sympatrically. To assess the reliability of morphological characters typically used to differentiate between these two species, we compared the concordance of species identification based on morphological characteristics with identifications based on a species-specific difference within the *avpr1a* gene. We found that intraspecific variation in morphological characteristics resulted in erroneous or ambiguous species identification in the field (generally ≤5%) as well as for preserved specimens (up to ~45%). Our data suggests that genotyping putative *M. ochrogaster* and *M. pennsylvanicus* at their *avpr1a* locus may be warranted for some individuals to ensure accurate species identification.

INTRODUCTION

In most ecological or behavioral studies, accurate species identification of the animals is critical. In the field, mammalogists typically use a combination of external morphological characters, such as pelage coloration and patterning, the length of the tail relative to the body, the length of the tail relative to the hind foot, the number of plantar tubercles and the number of mammae to distinguish between species (*e.g.,* Gottschang, 1981; Hall, 1981). However, in some cases, external morphology may be unreliable, either because the two species in question are extremely similar in appearance, or due to intraspecific variation in supposedly diagnostic morphological characters. For example, in a region of sympatry in the eastern United States, subspecies of *Peromyscus maniculatus* (deer mouse) and *P. leucopus* (white-footed mouse) are so morphologically similar that accurate species determination in the field is almost impossible, and a sizeable literature is devoted to methods for distinguishing between the two species (*e.g.,* Rich *et al.*, 1996; Bruseo *et al.*, 1999; Lindquist *et al.*, 2003). Where external morphology is unreliable, dental characteristics may allow the determination of species (*e.g.,* Hall, 1981). However, the observation of dental characters typically requires sacrificing the animal, making the use of dental characters unsuitable for studies in which the observation of individuals through time is necessary, such as many field studies that utilize catch-mark-recapture techniques. Thus, reliable identification of all individuals for some sympatric species may be extremely difficult in certain field situations.

When the identification of morphologically similar sympatric species in the field is difficult because of intraspecific variation or interspecific overlap in external morphology,

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species-specific differences at the molecular level may provide a more accurate means for identifying species. For example, Aquadro and Patton (1980) demonstrated that species-specific differences in salivary amylase electromorph phenotypes could be used to unambiguously distinguish between morphologically similar *Peromyscus leucopus* and *P. maniculatus*. More recently, several DNA-based techniques have utilized species-specific nucleotide polymorphisms to identify a variety of species of mammals (Berry and Sarre, 2007; O'Reilly *et al.*, 2007; Moran *et al.*, 2008). These molecular diagnostics have proven extremely useful in cases where it is difficult or impossible to distinguish between morphologically similar species in the field.

Microtus ochrogaster (prairie voles) and *M. pennsylvanicus* (meadow voles) are morphologically similar species of arvicoline rodents that occur sympatrically in some parts of the midwestern United States (Reich, 1981; Stalling, 1990). Numerous field studies have examined the ecology, demography and social behavior of these two species in areas of symapatry (*e.g.*, Krebs, 1977; Getz *et al.*, 2001, 2005), and positive species identification is critical. Unfortunately, intraspecific morphological variation may confound the ability of researchers to reliably identify the species of some individuals in the field using external characters as diagnostics (*e.g.*, DeCoursey, 1957; Fitch, 1957; Oppenheimer, 1965; Reich, 1981; Stalling, 1990; Schwartz and Schwartz, 2001).

The neuropeptide arginine vasopressin influences social and reproductive behavior in male *Microtus ochrogaster* and *M. pennsylvanicus* by directly regulating the neural expression of arginine vasopressin 1a receptors (V1aR; Lim *et al.*, 2004; Hammock and Young, 2005). Recent molecular studies have shown a species-specific difference between *M. ochrogaster* and *M. pennsylvanicus* in the length of microsatellite DNA within the gene (*avpr1a*) encoding the V1aR (Young *et al.*, 1999; Fink *et al.*, 2006) and the potential effects of this interspecific difference in *avpr1a* length on male social behavior have been extensively studied (Hammock and Young, 2005; Young and Hammock, 2007). *Microtus ochrogaster* have an expanded region of repetitive microsatellite DNA [approximately 430 base pairs (bp)] in the regulatory region of the *avpr1a* gene that is absent in *M. pennsylvanicus*. This length difference between species in the microsatellite DNA of the *avpr1a* gene has been found in *M. ochrogaster* sampled from populations in Illinois (IL), Kansas (KS) and Tennessee (Fink *et al.*, 2006; Ophir *et al.*, 2008; Solomon *et al.*, 2009) and *M. pennsylvannicus* populations from IL, Montana and Pennsylvania (Fink *et al.*, 2006). We used this apparent species-specific polymorphism in microsatellite length within the regulatory region of the *avpr1a* gene to determine species identity in order to assess the likelihood of species misidentification using the morphological characters typically employed for distinguishing between sympatric *M. ochrogaster* and *M. pennsylvanicus* in the field.

METHODS

Study areas and field procedures.—Our study sites were located at the University of Kansas' Nelson Environmental Study Area (NESA), 12 km northeast of Lawrence, KS (39°03′07″N, 95°11′27″W), and at the Indiana University Bayles Road Preserve (BRP), 5 km north of Bloomington, IN (39°13′00″N, 86°32′27″W). At each site, the study area was 1.5 ha, with live traps placed in a grid pattern and spaced at 10 m intervals. Both sites consisted of old-field habitat, containing a variety of grasses interspersed by patches of forbs, shrubs and tree seedlings and were mowed annually to discourage the growth of invasive species and woody vegetation. Only *Microtus ochrogaster* was present at the KS site, which is outside the species range of *M. pennsylvanicus* (Reich, 1981; Stalling, 1990; Slade, pers. comm.); both vole species occurred at the IN site.

TABLE 1.—Expected character state of morphological features considered diagnostic for distinguishing between *Microtus ochrogaster* and *M. pennsylvannicus*

Character	*M. ochrogaster*	*M. pennsylvanicus*
Plantar tubercles	5	6
Ventral pelage	yellow or rusty	gray or silver
Dorsal pelage	grayish brown; grizzled appearance	reddish brown; short, smooth fur
Tail length	<2× length of hind foot	>2× length of hind foot
Maxillar M^2	no posterior loop	posterior loop
Maxillar M^3	2 closed triangles	3 closed triangles

NOTE:—Diagnostic character states based on descriptions in Reich (1981), Gottschang (1981), Stalling (1990), and Schwartz and Schwartz (2001)

Fieldwork was conducted in KS during May–early Jul. 2008 and in IN during mid Jul.–Sept. 2008. At each site, voles were trapped twice daily, 4–5 d per week for 8 wk, using single-capture (Sherman; H.B. Sherman, Tallahassee, Florida) and multiple-capture (Ugglan; Grahnab, Hillerstorp, Sweden) live traps baited with cracked corn. Trapping occurred either at grid points (weeks 1, 2, 5 and 6) or known *Microtus ochrogaster* nest sites (weeks 3, 4, 7 and 8). Upon initial capture, all voles were given a unique toe-clip pattern for individual identification and the toes were frozen at −20 C for subsequent genetic determination of their *avpr1a* genotype.

All research procedures involving live voles followed the guidelines of the American Society of Mammalogists for the use of wild animals in research (Gannon *et al.*, 2007) and were approved by the Institutional Animal Care and Use Committees of Miami University, the University of Kansas, and Indiana University.

Assessment of species identity in the field.—For every capture event of a vole at the IN study site, a field assessment of species identity was made based on differences between *Microtus ochrogaster* and *M. pennsylvanicus* in number of plantar tubercles and pelage as described by Reich (1981), Gottschang (1981), Stalling (1990) and Schwartz and Schwartz (2001) and summarized in Table 1. During the course of the 8 wk study, field assessments of the species identity of voles were made by five people who differed in their level of experience in identifying *Microtus* species in the field.

Preserved specimens.—Differences in dental features of the maxillary molars are considered the most reliable diagnostic characters for distinguishing these two species of voles (Reich, 1981; Stalling, 1990; Schwartz and Schwartz, 2001). Since these dental characteristics can best be examined in preserved skulls, a subset of males (n = 15) were euthanized at the completion of fieldwork in IN to permit analysis of molar cusp patterns. In addition, preserved skulls of three *Microtus ochrogaster* and four *M. pennsylvanicus* were acquired from the Miami University Hefner Zoology Museum in Oxford, OH. All seven voles from the museum were collected in the area around Oxford, OH. Molar cusp patterns of these 22 skulls were viewed through a dissecting microscope. The diagnostic character states of the second (M^2) and third (M^3) maxillae molars for each species are summarized in Table 1, as described by Reich (1981) and Stalling (1990).

The number of plantar tubercles and the ratio of tail length:hind foot length from field-collected specimens was also recorded. *Microtus ochrogaster* typically have five plantar tubercles and a tail length:hind foot length ratio <2, while in *M. pennsylvanicus* the typical number of plantar tubercles is six with a tail length:hind foot length ratio >2 (Table 1; Schwartz and Schwartz, 2001). When data on tail and hind foot lengths were available from specimen tags of preserved skulls, this information from museum specimens was also recorded. We only present data on the tail length/hind foot length ratio for the males collected from the IN site and the museum specimens because of the difficulty of accurately measuring these characters in living animals. We were unable to determine the number of plantar tubercles from museum specimens because we only had access to the skulls. All of the data on dentition, plantar tubercle number and tail length:hind foot length ratio of the preserved specimens was collected by one of the authors (ACH). The *avpr1a* genotypes of the museum specimens were determined from tissue collected by scraping the interior of brain cases with a sterilized sewing needle.

avpr1a genotyping.—Genomic DNA was extracted using DNeasy kits (Qiagen, Valencia, CA) and the *avpr1a* genotypes of voles were determined by polymerase chain reaction (PCR), with primers specifically designed to amplify the microsatellite region of the *avpr1a* gene in prairie voles (Hammock and Young, 2005). Our *avpr1a* genotyping procedure was identical to that followed by Solomon *et al.* (2009), who confirmed that these primers amplified the targeted region of the *avpr1a* gene by sequencing the resulting PCR product and comparing the sequence to the prairie vole *avpr1a* gene sequence published in GenBank. Previous studies using the same primers as in our investigation have demonstrated that PCR amplification of this microsatellite region of the *avpr1a* gene in known *Microtus pennsylvanicus* yields PCR fragments that are 200–300 bp, while in known *M. ochrogaster* the resultant PCR fragments are 600–800 bp (Young *et al.*, 1999; Fink *et al.*, 2006).

Data analysis.—All means are reported ± 1 SE. For the voles from the IN field site, we compared the concordance of species identification based on morphological characteristics observable in the field (pelage and plantar tubercles) with identifications based on *avpr1a* genotypes for all adult male voles trapped only once and all adult males trapped four or more times. Thus, we used two different levels of contact with live-trapped voles. Data from animals that were only trapped once were used to assess the accuracy of species identification when each animal was observed by a single handler. Data from animals with ≥4 captures increased the chances that an individual vole would be identified by multiple observers and allowed us to examine if capture history may be helpful in clarifying misidentification issues.

RESULTS

Species identity in the field.—We determined the *avpr1a* genotypes of 70 adult males from the KS study site and 279 adult males from the IN study site. At the KS site, where only *Microtus ochrogaster* occurs, allele lengths ranged from 686 to 797 bp (Fig. 1). The distribution of *avpr1a* allele lengths at the IN site, where both vole species occur, was bimodal, with 50 individuals possessing two alleles ranging from 289 to 294 bp and 229 voles possessing two alleles between 696 and 798 bp (Fig. 1). None of these voles possessed one allele between 289–294 bp and the other between 696–798 bp. Thus, all the voles from the KS site as well as the 229 voles from the IN site with *avpr1a* allele lengths between 696 and 798 bp had *avpr1a* genotypes diagnostic of *M. ochrogaster,* while the 50 voles at the IN site with alleles lengths between 289 and 294 bp had *avpr1a* genotypes diagnostic of *M. pennsylvanicus*. The *avpr1a* genotype data from IN included the 15 males collected from the population at the end of the study, of which 13 had *M. ochrogaster avpr1a* genotypes and two had *M. pennsylvanicus avpr1a* genotypes.

Voles trapped only once.—There were 58 male voles at the IN site that were captured only once. Based on their *avpr1a* genotypes, 17 of these voles were *Microtus pennsylvanicus* and 41 were *M. ochrogaster*. Among the voles with *M. pennsylvanicus avpr1a* genotypes, 5 (29%) were identified in the field as *M. ochrogaster*. All five of these individuals had five plantar tubercles

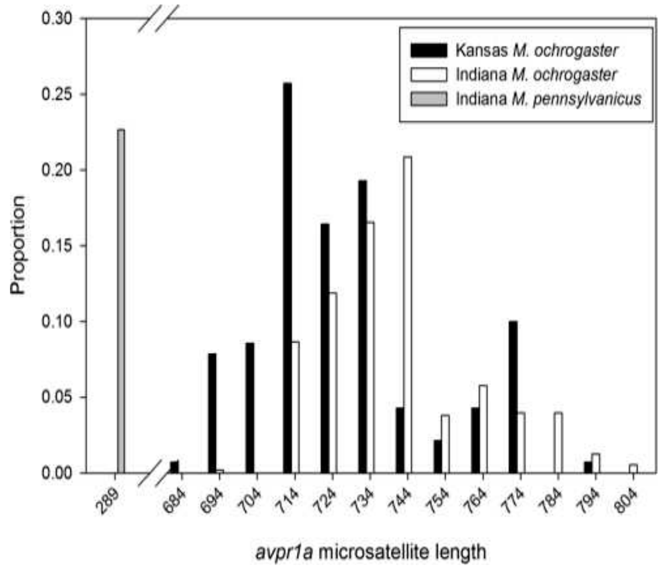

FIG. 1.—Frequency distribution of *avpr1a* microsatellite allele lengths (bp) among adult males in KS (n = 70) and IN (n = 279). Individuals from Indiana with *avpr1a* genotypes characteristic of *Microtus pennsylvanicus* are shown in gray. Note the 380 bp break in the x-axis

as is expected for *M. ochrogaster*. For the voles with *M. ochrogaster avpr1a* genotypes, one individual (2.5%) was identified as *M. pennsylvanicus* and had six plantar tubercles that is characteristic of *M. pennsylvanicus*. An additional vole (2.5%) identified as *M. pennsylvanicus* had an indeterminate number of plantar tubercles, and was later genetically identified as *M. ochrogaster*. Overall, 12% (7/58) of the voles captured only once were misidentified in the field and in six of the seven cases of misidentification the vole had the number of plantar tubercles characteristic of the species for which it was mistaken.

Voles trapped four or more times.—For the 221 voles caught four or more times at the IN site, voles with *Microtus ochrogaster avpr1a* genotypes were misidentified in 4.9% of all capture events, and animals with *M. pennsylvanicus avpr1a* genotypes were incorrectly identified in 4.2% of all capture events. Considering the total capture history of each individual, 28% (52/188) of individuals with *M. ochrogaster avpr1a* genotypes were recorded as *M. pennsylvanicus* at least once. Similarly, 30% (10/33) of voles with *M. pennsylvanicus avpr1a* genotypes were identified as *M. ochrogaster* at least once. Thus, 28% (62/221) of all adult male voles trapped $\geq$4 times at the IN site were misidentified to species during at least one capture. In 87% of these cases the number of plantar tubercles recorded for the misidentified animal was characteristic of the species that it was incorrectly identified as in the field.

Preserved specimens.—Of the 22 vole skulls examined, one of the 16 (6%) voles with a *Microtus ochrogaster avpr1a* genotype deviated from the expected dental characteristics for this species, possessing an atypical molar cusp pattern at M^3 that did not resemble the expected pattern of either species (Fig. 2). Among the six voles with a *M. pennsylvanicus avpr1a* genotype, one individual (17%) deviated from the expected dental features for this species at both M^2 and M^3, possessing typical ''*ochrogaster*'' M^2 morphology and irregular M^3

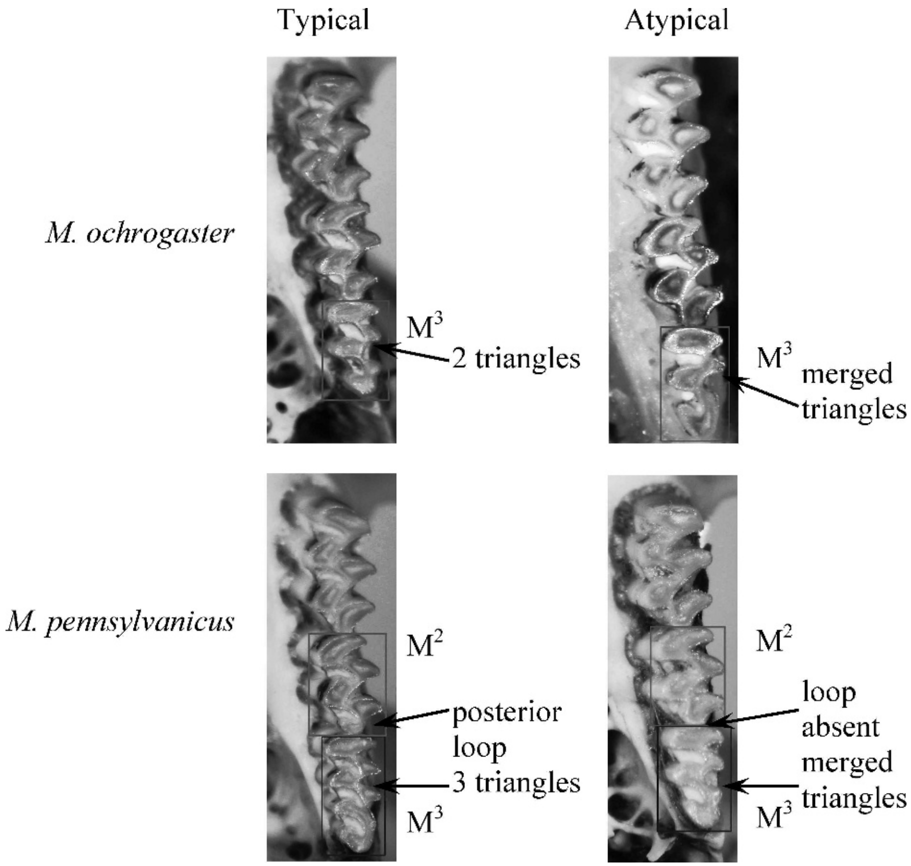

FIG. 2.—Examples of typical and atypical maxillae molar dentition in *Microtus ochrogaster* and *M. pennsylvanicus.* Molars of the left upper maxillae are oriented anteriorly towards the top of each photo

morphology (Fig. 2). Overall, the molar morphology of 9% (2/22) of skulls examined did not match that expected based on their *avpr1a* genotype.

The average tail length/hind foot length ratio among voles collected from the IN site and the museum specimens was 1.96 ± 0.1 (n = 14) for individuals with *Microtus ochrogaster avpr1a* genotypes and 2.19 ± 0.1 (n = 4) for individuals with *M. pennsylvanicus avpr1a* genotypes. Only 57% (8/14) of these voles with *M. ochrogaster avpr1a* genotypes had tail length/hind foot length ratios less than two, as expected for this species (Table 1; Schwartz and Schwartz, 2001). For voles with *M. pennsylvanicus avpr1a* genotypes, 25% (1/4) had tail length/hind foot length ratios characteristic (<2) of *M. ochrogaster.* Among all the preserved vole specimens, 39% (7/18) of the individuals had tail length/hind foot length ratios that were inconsistent with their species diagnostic *avpr1a* genotypes.

For the males collected from the IN site, only one of 13 voles (8%) with a *Microtus ochrogaster avpr1a* genotype had an atypical number of plantar tubercles (six rather than five), while both of the voles with *M. pennsylvanicus avpr1a* genotypes had the species diagnostic six plantar tubercles. Overall 7% (1/15) of these voles had an atypical number of plantar tubercles.

When considering dentition, tail length/hind foot length ratio and plantar tubercles together, nine of the 22 preserved voles (41%) had one morphological feature that exhibited a character state that was not consistent with their *avpr1a* genotype and one vole (5%) had two morphological features that displayed character states not consistent with its *avpr1a* genotype. Thus, 46% of the preserved voles had at least one morphological feature exhibiting a character state that was inconsistent with their *avpr1a* genotype.

Discussion

Our *avpr1a* genotype data for the voles we sampled from KS and IN were completely consistent with the previously reported species-specific genetic polymorphism in *avpr1a* allele length between *Microtus ochrogaster* and *M. pennsylvanicus*, with *M. ochrogaster* containing an expanded segment of repetitive microsatellite DNA in the *avpr1a* gene that is absent in *M. pennsylvanicus* (Young *et al.*, 1999; Fink *et al.*, 2006). Since only *M. ochrogaster* occurs at our KS study site, we expected and found all the individuals we sampled to have two *avpr1a* alleles between approximately 600 and 800 bp in length. At our IN study site, where the two vole species are sympatric, all individuals had two *avpr1a* alleles characteristic of either *M. ochrogaster* (696–798 bp) or *M. pennsylvanicus* (289–294 bp). No voles possessed *avpr1a* microsatellite lengths that were characteristic of both species, or atypical for either species. Therefore, we were able to use *avpr1a* allele lengths to unambiguously identify the species of all adult male voles from the IN study site genotyped at this locus.

Although pelage and number of plantar tubercles are the primary phenotypic characters used for distinguishing between *Microtus ochrogaster* and *M. pennsylvanicus* in the field, we found that, in our hands, they were not completely reliable when compared to the species diagnostic *avpr1a* genotypes of individuals. Species misidentification at our IN site ranged from ~5% per capture event for *M. ochrogaster*, regardless of the number of times captured, to 29% for *M. pennsylvanicus* captured only once. Categorizing pelage color is very subjective, and subject to inter-observer differences. Moreover, both species display intraspecific variability in pelage color within and between populations (Gottschang, 1981; Reich, 1981; Stalling, 1990). We found some individuals with *M. ochrogaster avpr1a* genotypes to possess *M. pennsylvanicus*-like pelage color, and vice versa. While plantar tubercle number is a quantitative character and less subjective, it is not invariant in either vole species (Fitch, 1957; Schwartz and Schwartz, 2001). We found that for almost 90% of the cases of species misidentifications in the field, the misidentified vole had an atypical number of plantar tubercles. In addition, the number of plantar tubercles on an individual may be ambiguous due to wear, differential pigmentation or the amount of dirt on the feet.

It might be expected that preserved specimens of *Microtus ochrogaster* and *M. pennsylvanicus* would be less prone to species misidentification since it should be easier to examine species diagnostic morphological characters, including dentition, on these voles than individuals in the field. However, for the three morphological characters we examined, 41% of the preserved specimens had one character state indicative of *M. ochrogaster* and another characteristic of *M. pennsylvanicus*, making species identification of these individuals ambiguous (one vole had dentition atypical for either species).

Features of the second and third maxillary molars are considered the most reliable morphological character for distinguishing between *Microtus ochrogaster* and *M. pennsylvanicus* (Reich, 1981; Stalling, 1990; Schwartz and Schwartz, 2001), but even with our small sample size (n = 22) we encountered two (9%) instances where molar morphologies deviated from that expected based on the *avpr1a* genotype of the animals. Interestingly, the percentage of voles where the expected number of plantar tubercles was not consistent with an individual's *avpr1a* genotype was similar to that for molar dentition (7%), suggesting that plantar tubercle number may be as reliable a character as dentition for species identification. The tail length/hind foot length ratio was the least reliable diagnostic morphological character we examined as 43% of the voles with *M. ochrogaster avpr1a* genotypes and 25% of voles with *M. pennsylvanicus avpr1a* genotypes deviated from their expected ratio. These results suggest that the tail length/hind foot length ratio would be even less reliable when used in the field, when measurements must be made on live animals.

The data from our study demonstrates that using morphological characteristics to distinguish between *Microtus ochrogaster* and *M. pennsylvanicus* results in cases where species identity is erroneous or ambiguous. Although genotyping voles at their *avpr1a* locus is more time consuming and expensive than using morphological characters, we believe there are situations where such genetic analysis may be warranted to obtain accurate species identification of all individuals where *M. ochrogaster* and *M. pennsylvanicus* are sympatric. The species identity of voles caught in the field ≥4 times was misidentified in 4–5% of all capture events, but for these animals an individual's capture history may be used to identify, and possibly correct, cases of species misidentification given the low error rate. Approximately 5% of *M. ochrogaster* and 29% of *M. pennsylvanicus* captured only once were misidentified. In particular, the level of misidentification of *M. pennsylvanicus* captured only once was substantial and 7–8 times that of *M. pennsylvanicus* captured ≥4 times. Determining the *avpr1a* genotype of voles captured just once may be the only way to detect instances of species misidentification and ensure the accurate species identification of all individuals. Our data indicate that species identification is not just a problem in mark–recapture field studies. The species identity of about 40% of the preserved specimens we examined exhibited diagnostic characteristics of both *M. ochrogaster* and *M. pennsylvanicus,* but any ambiguity about the species identity of these specimens could be eliminated by genotyping these animals at the *avpr1a* locus. In general, we suggest that genotyping putative *M. ochrogaster* and *M. pennsylvanicus* at their *avpr1a* locus can be a useful diagnostic for eliminating erroneous or ambiguous species identification in many studies.

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