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Research Article

Moving north: Morphometric traits facilitate monitoring of the expanding steppe whiskered bat *Myotis davidii* in Europe

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Abstract

Various bat species are expanding their ranges due to changes in climate and landscape. These range expansions should be monitored thoroughly because they may alter local bat communities. The steppe whiskered bat *Myotis davidii*, for instance, has probably expanded its range from Central Asia to Eastern Europe. However, monitoring the range expansion of *M. davidii* is challenging because *M. davidii* and its sister species *M. mystacinus* are morphologically similar. Here, we investigated whether *M. davidii* occurs in Austria, which would extend its known range to the North-West. To facilitate the morphological identification of *M. davidii* and enable efficient monitoring approaches, we propose a morphometric approach. We analysed morphometric data of 102 *M. mystacinus* and 78 *M. davidii*. We applied sex-specific linear discriminant analyses to investigate whether a combination of hindfoot length, tibia length and forearm length could be used to distinguish *M. davidii* from *M. mystacinus*. The discriminant functions correctly identified 88 % of females and 82 % of males of the genetically verified individuals. Combined with dental characteristics, bat workers can reliably identify *M. davidii* based on morphometric traits. To investigate whether *M. davidii* had been previously found in Austria, we applied the discriminant functions to data of 61 Austrian *M. mystacinus* specimens preserved in the Natural History Museum Vienna. Since we did not find *M. davidii* specimens in the museum's collection – the most comprehensive of Austrian mammal collection – we presume that *M. davidii* is a relatively new element of the Austrian fauna. This indicates that *M. davidii* has expanded its range over the last decades. The discriminant functions will facilitate monitoring of this potential range expansion.

Introduction

Recent changes in climate and landscapes can lead to shifts in the distribution of animal species (Chen et al., 2011; Maclean et al., 2008). At the cooler border of their ranges, warm-adapted animal species may profit from warming environments and may expand their ranges into previously cooler regions, while some parts of a species' current distribution range may become unsuitable due to global warming (Ancillotto et al., 2016). In European bats, for instance, both Kuhl's pipistrelle *Pipistrellus kuhlii* and Savi's pipistrelle *Hypsugo savii* have expanded their range substantially during the last decades towards the north; probably caused by changes in climate and landscapes (Ancillotto et al., 2016; Uhrin et al., 2016; Reiter et al., 2010).

Detecting and monitoring changes in the distribution of species is necessary because these changes could entail new competitive interactions between newcomers and the local communities (Greenfeld et al., 2018). *Pipistrellus kuhlii*, for instance, is thought to outcompete the common pipistrelle *P. pipistrellus* at foraging sites in recently exploited regions (Salinas-Ramos et al., 2021). Competitive interactions between *Hypsugo savii* and *P. pipistrellus* are also expected, yet remain to be investigated (Smeraldo et al., 2021). Range shifts and changes in local bat communities, where species can be easily identified, can be reliably studied. This is the case for *P. kuhlii*, and *H. savii* thanks to their unequivocal morphological traits in Central Europe. In contrast, cryptic bat species groups provide more significant challenges because the species are morphologically very similar. For instance, the whiskered bat species complex includes four morphologically similar species in Europe: whiskered bat *Myotis mystacinus*, Alcathoe bat *M. alcathoe*, Brandt's bat *M. brandtii*, and steppe whiskered bat *M. davidii*, formerly known as *M. aurascens* (Benda et al., 2012). Even experienced bat workers struggle with the morphological identification of these species (Lučan et al., 2011). Thus, potential changes in the distribution of these species can easily be overlooked.

A recent phylogeographic study of Eurasian whiskered bats suggests a cryptic range expansion of *M. davidii* from Asia towards the West, where they came in contact with *M. mystacinus* across a wide geographic area (Çoraman et al., 2020). Within the areas of sympatric distribution in Anatolia and the Balkans several independent introgression events of the mitochondrial genome of *M. mystacinus* into *M. davidii* were documented (Çoraman et al., 2020). Mitochondrial introgression happens more likely during the expansion of a species because the invader is rather rare, which impedes mating among conspecifics (Hubbs, 1955). Mitochondrial introgression was documented for several other species pairs such as *M. myotis* and *M. blythii*, where *M. blythii* invaded the range of *M. myotis* (Berthier et al., 2006) or *Eptesicus nilssonii* and

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E. serotinus, where *E. serotinus* invaded the range of *E. nilssonii* (Artyushin et al., 2009; Mayer et al., 2001).

Mitochondrial introgression makes species identification unreliable if it is only based on sequencing a part of the mitochondrial genome. Investigating the nuclear genome would resolve this problem, but reliable genetic markers are usually lacking. Therefore, unequivocal morphological traits are needed to study the ecology of a species and, for example, to trace the current range expansion of *M. davidii*. While distinguishing *M. davidii* from both *M. brandtii* and *M. alcathoe* is straightforward due to different dental characteristics, *M. davidii* and *M. mystacinus* lack evident morphological determination criteria so far (Dietz et al., 2016). However, Dietz et al. (2016) noticed that the hind feet and tibiae of *M. davidii* are conspicuously larger than those of *M. mystacinus*. Thus, these morphological traits might provide valuable determination criteria.

Here, we report the first records of *M. davidii* in Austria, which extends the range of this species to the North. Furthermore, we tested whether morphological traits are reliable to distinguish *M. davidii* from *M. mystacinus* already in the field. Specifically, we built linear discriminant functions (LDF) based on morphological data of 102 *M. mystacinus* and 78 *M. davidii*. Whole genome sequencing was performed on 15 individuals representing both species in order to evaluate morphological species identification. Other individuals were identified based on two to four nuclear introns by Çoraman et al. (2020). To investigate whether *M. davidii* had been previously recorded in Austria, we applied the LDF on 61 Austrian *M. mystacinus* specimens from the Natural History Museum Vienna.

Materials and methods

Bat capture

We captured bats in the Natura 2000 site Lendspitz-Maiernigg (site code: AT2130000) in southern Austria (46°36′ N, 14°26′ E, WGS84). This Natura 2000 site comprises an area of 77.6 ha and is located in the Carinthian city of Klagenfurt at Lake Wörthersee within the Klagenfurt basin. The Natura 2000 site covers the last natural shore of Lake Wörthersee, characterised by wetland habitats such as wet meadows, fens and reeds, and deciduous forests such as Luzulo-Fagetum beech forests and Illyrian oak-hornbeam forests Glatz-Jorde et al. (2016). The Klagenfurt basin is characterised by a temperate oceanic climate according to the Köppen climate classification (9 °C mean annual temperature, 1332 mm mean annual precipitation).

Bats were captured at two sites during eight sampling nights from 2019 to 2021. Site 1 was located at the northern edge of the Natura 2000 site on former tramway tracks. Hair nets and monofilament nets (mesh size 14×14 mm^2, Ecotone Goc, Sopot, Poland) were placed within a tunnel-shaped vegetation structure for six sampling nights. Site 2 was located at a bridge over the Sattnitz, a small river runoff of Lake Wörthersee. We placed one monofilament net under the bridge close to the water surface during two sampling nights. Capturing bats was conducted under license BG-NR/43/2014, 08-NATP-845/11-2020(004/2021) and BG-NR/00269/2021/01.

Morphological identification of captured *Myotis sp*

To pre-identify *M. davidii* morphologically, we followed the notes of Benda et al. (2012) and Dietz et al. (2016). According to the authors, *M. davidii* exhibits small and indented upper P^3, colourful fur, bright faces, and large hind feet. Thus, we recorded these qualitative traits in all captured *M. cf. davidii* and *M. cf. mystacinus*. In addition to the qualitative characteristics, we recorded forearm length, hindfoot length and length of tibia (measurements details in Dietz et al., 2016, 133 p). We recorded both the sex and age of all captured bats. The age was estimated in two classes based on the ossification of their digits. Bats with long stretched joints, cartilaginous fingers or visible growth plates near the joints were classified as subadults. Individuals with fully ossified digits were classified as adults.

Genomic analysis

For the genomic analysis, we took small tissue samples from the uropatagium (license: 10-TVG-26/2-2020) because it has been shown to heal faster than the wing membrane (Faure et al., 2009). We stored the tissue in 96 % ethanol. Subsequently, we randomly selected four *M.* cf. *davidii* and one *M.* cf. *mystacinus* for genomic species assignment.

Morphological species identification was confirmed by whole genome sequencing of four *M. davidii* and one *M. mystacinus* from the sympatric locality in the Klagenfurt basin of Carinthia plus five *M. mystacinus* from Central Europe and five *M. davidii* from the southern Balkan peninsula Fig. 1B. Genomic DNA was isolated with a salt-chloroform extraction method Müllenbach et al. (1989) and sheared by a S220 Focused-ultrasonicator (Covaris Inc., Woburn, USA) to achieve a fragment length below 800 bp. Libraries for whole genome shotgun sequencing were prepared by using NEXTflex Rapid XP DNASeq Kit (PerkinElmer Inc., Waltham, USA) following the manufacturer's manual. 150 bp paired-end sequencing took place on a NextSeq500 sequencing system (Illumina Inc., San Diego, USA). On average, we received 132 Million 150 bp reads per sample, ranging from 78 to 187 Million reads. The raw sequence reads were filtered using the nf-polish pipeline (https://github.com/MozesBlom/nf-polish, accessed on 11/11/2022), by which 21.6 % of reads were removed. The remaining reads were mapped against the *M. myotis* reference genome (NCBI RefSeq GCF_014108235.1) (Jebb et al., 2020) using the nfmap pipeline (https://github.com/MarieGurke/nfmap, accessed at 11/11/2022). This resulted in an average coverage of 6.2x (range: 3.5x-8.7x). We used angsd version: 0.935 (Korneliussen et al., 2014) to calculate genotype likelihoods from the mapped data and applied the following filters: SNP p-value of below 1e-6, minimum base calling the quality of 20, minimum mapping quality of 20 and a maximum depth of 12. The genotype likelihoods at 1,382,061 variant sites (SNPs) were then used to do a principal component analysis (PCA) using the program PCAngsd v.1.10 (Meisner et al., 2018).

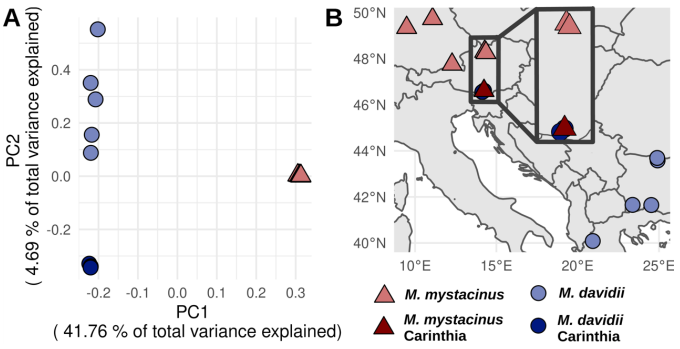

Figure 1 – (A) PCA scatter plot *M. davidii* (blue) and *M. mystacinus* (red) samples from within the study region Carinthia (dark colours) and outside of it (light colours). All samples of *M. mystacinus,* as well as the *M. davidii* samples from Carinthia are overlapping. B) shows a map with the geographic samples locations of the sample used for the PCA shown in (A). Grey rectangles zoom in on areas of both plots where sample points are overlapping.

Statistical analysis

For our statistical analyses, we used the morphometric data (i.e. hind feet length, tibia length, forearm length) of the nine *M. davidii* and six *M. mystacinus* that were identified through whole genome sequencing. Additionally, we used morphometric data of 69 *M. davidii* captured or collected at 16 sites in five European countries (Bulgaria, Croatia, Greece, Slovenia, Turkey, Fig. 2) and measured by field workers. Measurements and the age of those individuals were determined according to the individuals captured in Carinthia. To verify the morphological identification of individuals outside our main study site in Carinthia, we used a population-level approach, where at least one or two individuals from each local population were randomly selected for sequencing. Local populations were defined as maternity roosts or capture sites. The individuals were analysed by sequencing two to four nuclear

introns of wing membrane samples (i.e. intron 5 of ABHD11 gene, intron 3 of ACOX2 gene, intron 4 of COPS gene, intron 7 of ROGDI gene) in Çoraman et al. (2020). Additionally, we analysed measurements from 96 *M. mystacinus* specimens collected from 45 locations in France and Germany. Of 26 randomly selected populations, identification as *M. mystacinus* was confirmed by sequencing two to four nuclear introns of wing membrane samples. In addition, given that *M. davidii* is neither known nor expected in Germany and France, and the closest record of *M. davidii* was located 283 km away from these countries, we concluded that the measurement values were accurately assigned to this species. Individuals confirmed through genomic analysis but lacking complete measurement data were excluded from the morphometric analysis. A list of all individuals used in the morphometric analysis is provided in Table S1 in the Supplementary material.

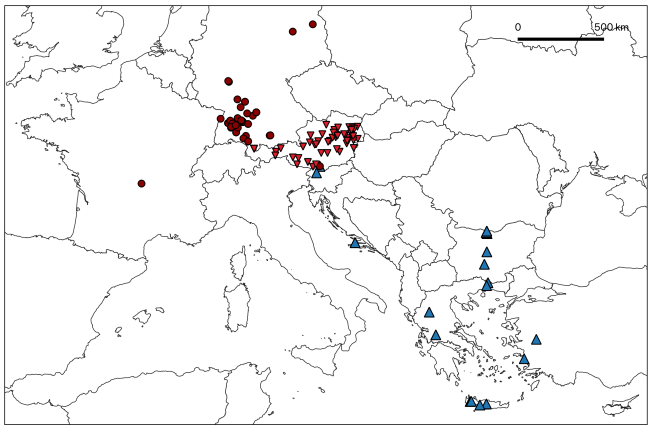

Figure 2 – Locations of analysed individuals of *M. mystacinus* and *M. davidii* in Europe, verified by whole genome sequencing or two to four nuclear introns. The red reverse triangles show 61 locations of *M. mystacinus* specimens preserved in the collection of the Natural History Museum Vienna. The blue triangles are the locations of the analysed *M. davidii* and the green dots indicate the locations of the analysed *M. mystacinus*.

We followed a two-step approach to investigate whether morphometric data can be used to distinguish *M. davidii* from *M. mystacinus*. In the first step, we applied two MANOVAs for each species to test whether sex, age or biogeographical region influenced the morphometric data. Biogeographical regions were defined following the definitions of the European Environmental Agency (Roekaerts, 2002) where the individuals originated from (i.e. Continental, Alpine, Mediterranean and Crete). Crete was separated from the Mediterranean region in the analysis because insular bats are often differently sized compared to mainland bats (Lomolino, 2005). Before the MANOVA analyses, we checked for multivariate normality using Shapiro-Wilk tests, for homogeneity of variance-covariance matrices using Box's M-test (Box G.E.P., 1949), and for multicollinearity among the morphometric measures by calculating Pearson's r among all measures. We used the R packages heplots (Fox et al., 2021) and corrplot (Wei et al., 2021) to check those assumptions. We included forearm length, hindfoot length and tibia length as dependent variables in the MANOVAs, and sex, age and region as independent variables. Subsequently to significant MANOVA results, we applied univariate ANOVAs to each dependent variable to identify the dependent variables contributing to the significant MANOVA effects. We then determined statistically significant groups using Tukey post hoc tests on the univariate ANOVA results.

In a second step, we applied linear discriminant analyses using the R package MASS (Venables et al., 2002.) to distinguish *M. mystacinus* from *M. davidii*. Following the results from the MANOVA analyses, we applied three models: one to both sexes (from now on global LDF), one to male individuals and one to female individuals (from now on sex-specific LDF). Cretan individuals (n=8)were excluded from this analysis step because they were significantly smaller than mainland individuals. We validated the models using Leave-one-out cross-validation and calculated cross-tabulations using the R package caret (Kuhn, 2021).

To investigate if *M. davidii* has been found in Austria previously, we used the determined sex-specific LDF on morphometric data of 61 *M.* cf. *mystacinus* specimens collected by the Natural History Museum Vienna at 51 locations in Austria to investigate whether *M. davidii* has been previously recorded in Austria. To ensure reliable measurements, we only measured museum specimens whose hind feet were straightened during preparation. All statistical analyses were conducted in R 4.1.3 (R Core Team, 2022).

Results

Morphological identification

We captured 100 bats of 12 species in eight sampling nights in our study area of Carinthia (Tab. 1). 20 individuals with similar morphological traits were pre-identified as *M. davidii* and seven as *M. mystacinus.* The dorsal furs of *M.* cf. *davidii* were golden with a distinct contrast to the white ventral hairs. The face and the base of the ears were brightened, similar to *M. brandtii.* The upper P^3 were tiny and palatally indented; the upper P^4 did not have a cingular cusp. Paraconuli were absent on all upper molars. The lower P_3 were less than a third the size of the lower P_2. Detailed pictures are provided in Fig. S1, Fig. S2 and Fig. S3 in the Supplementary material.

Table 1 – Captured bat species at two sites within the Natura 2000 area Maiernigg-Lendspitz, capture year and the number of individuals. Site 1 refers to the tramway tracks and site 2 to the bridge over the Sattnitz river.

Species	2019	2020	2021	Total	Sites
Myotis daubentonii	0	0	2	2	2
Myotis mystacinus	4	2	1	7	1
Myotis davidii	0	6	14	20	1 and 2
Myotis bechsteinii	0	0	1	1	1
Myotis crypticus	1	0	0	1	1
Myotis emarginatus	1	3	0	4	1
Myotis myotis	1	0	0	1	1
Pipistrellus pipistrellus	11	8	4	23	1
Pipistrellus pygmaeus	8	4	4	16	1
Pipistrellus kuhlii	0	0	2	2	1
Pipistrellus nathusii	0	0	3	3	1
Barbastella barbastellus	9	6	5	20	1

Genomic analysis

The Principal Component Analysis of the whole genome data sets reveals two clearly distinct groups (Fig. 1A). Samples of the two species *M. mystacinus* and *M. davidii* are clearly separated. The first principal component (x-axis) explains 41.8 % of the variability and clearly separates *M. mystacinus* and *M. davidii*. All animals of the study area in Carinthia clearly group either *M. mystacinus*, respectively *M. davidii* from other regions and thus confirm species identification based on external morphological characters. The second principal component (y-axis) explains only 4.7 % of the genomic variability and separates *M. davidii* samples from Austria from those collected in the Balkans.

Morphometric analysis

In both *M. mystacinus* and *M. davidii*, sex had a significant effect on the combined morphometric traits (Pillai's trace=0.16, F (3, 95)=6.84, p<0.001 and Pillai's trace=0.22, F(3,66)=4.83, p<0.01, respectively), while age had no significant effect. Univariate ANOVAs with Bonferroni adjusted significance levels of 0.02 showed that forearms were significantly longer in females than in males (*M. mystacinus*: F(1,99)=11.8, p<0.001) and *M. davidii*: F(1,72)=9.83, p<0.01). The biogeographical region did not significantly influence the measures in *M. mystacinus* but was highly significant in *M. davidii* (Pillai's trace=0.6, F(9,204)=5.05, p<0.00001). Subsequent univariate AN-

OVAs with Bonferroni adjusted significance levels of 0.02 showed significant regional differences for both hind foot length ($F(3,70)=11.6$, $p<0.00001$) and forearm length ($F(3,70)=10.4$, $p=0.00001$). Post hoc Tukey tests revealed that, as expected, Cretan *M. davidii* had significantly shorter forearms and hind feet than mainland *M. davidii*.

The global LDF calculated from tibia length, hindfoot length and forearm length correctly classified 84 % of *M. davidii* and *M. mystacinus*. In the sex-specific LDF, 88 % of females and 82 % of males were classified correctly (Fig. 3). In all models, both hindfeet and tibiae were significantly longer in *M. davidii*, while forearm length contributed least to the classifiers (Tab. 2, Fig. 4).

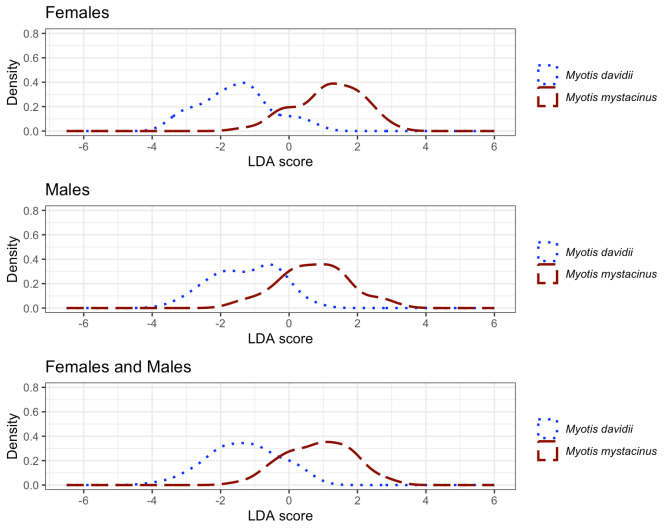

Figure 3 – Linear discriminant analysis (LDA) scores of three models aiming to distinguish *M. davidii* from *M. mystacinus*: we analysed the performance of hindfoot length, tibia length and forearm length as discriminators between *M. davidii* and *M. mystacinus*. The upper figure shows the LDA scores of the female-specific model, the middle of the male-specific model, and the lower of the global model (i.e. both sexes combined). Dotted lines indicate *M. davidii*, long-dashed lines *M. mystacinus*.

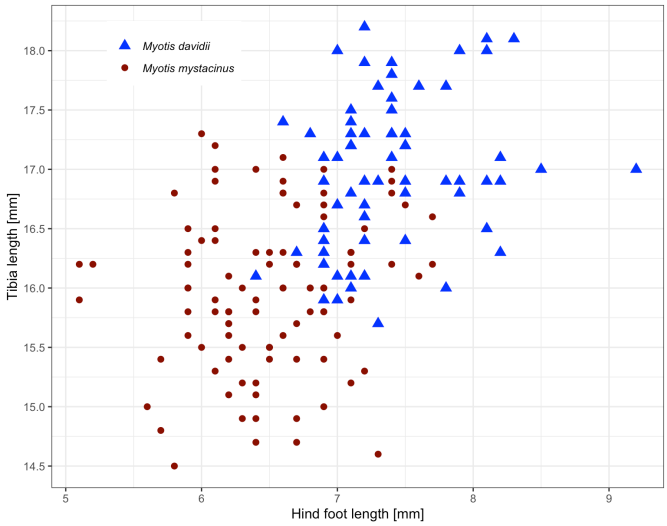

Figure 4 – Differences in hindfoot length and tibia length between European *M. davidii* and *M. mystacinus* individuals. We measured and plotted hindfoot length and tibia length of 78 *M. davidii* and 102 *M. mystacinus*. Points indicate *M. mystacinus*, triangles *M. davidii*.

The sex-specific LDF suggests 95 % of the museum specimens as *M. mystacinus*. The remaining three individuals were assigned to *M. davidii*. However, the mean posterior probability of these three individuals was a third lower than that of the specimens classified as *M. mystacinus* (0.61 vs 0.92, respectively). We checked both dental characteristics and fur colour of the three museum specimens classified as *M. davidii*. None of them showed an indented P^3, nor distinct contrasts between golden dorsal furs and white ventral furs. Thus, we assumed they were indeed *M. mystacinus*.

Table 2 – Coefficients of linear discriminants (i.e. tibia length, hindfoot length, forearm length) of the three linear discriminant analyses (LDA) and the respective True skill statistics (TSS).

LDA	Tibia length	Hind foot length	Forearm length	TSS
Global	−1.23	−1.32	0.32	0.67
Females	−1.93	−0.92	0.62	0.76
Males	−0.71	−1.65	0.14	0.59

Discussion

We report the first records of *M. davidii* in Austria. Initial morphological species identification of *M. davidii* and *M. mystacinus* was verified by whole genome sequencing. Our analysis of 78 *M. davidii* and 102 *M. mystacinus* shows that, in most cases, hindfoot length, tibia length, and forearm length allow European *M. davidii* to be distinguished from *M. mystacinus*.

The observed morphometric differences between *M. davidii* and *M. mystacinus* support Dietz et al. (2016), who report long tibiae and hind feet in *M. davidii* compared to those in *M. mystacinus*. Benda et al. (2016), on the other hand, showed high morphological overlaps between Caucasian *M. davidii* and *M. mystacinus*, which contradicts our approach toward morphological identification of *M. davidii*. However, Benda et al. (2016) only used mitochondrial markers to identify specimens. Hybridisation between *M. mystacinus* and *M. davidii* has led to multiple mitochondrial introgression events from *M. mystacinus* into *M. davidii* (Çoraman et al., 2020). Consequently, the mitochondrial DNA of *M. davidii* is often similar to that of *M. mystacinus*, and thus, identifying *M. davidii* based on mitochondrial markers might lead to wrong species identification. We, therefore, assume that partially wrong species assignment contributed to the morphological overlap between *M. mystacinus* and *M. davidii* reported by Benda et al. (2016). Since we focus our study on European individuals, we recommend testing our morphometric approach with Caucasian populations to validate this assumption.

In our morphometric models, hind feet and tibiae were significantly longer in *M. davidii* and contributed best to the LDF. The extended hind feet and tibiae in *M. davidii* may reflect their foraging behaviour. Trawling bats which forage close to water surfaces, like the Daubenton's bat *M. daubentonii* or the long-fingered bat *M. capaccinii*, show extended hind feet to capture insects from the water surface (Fenton et al., 2002). Since we captured most *M. davidii* under a bridge close to the water surface, we suppose they frequently forage close to the water surface. This complies with observations by Chung et al. (2013), who showed that *M. davidii* prefers water bodies for foraging in South Korea and Zhigalin (2019), who captured 25 *M. davidii* close to river surfaces in Siberia and the Urals. On the other hand, *M. mystacinus* is associated with terrestrial foraging habitats, ranging from forests to open areas (Bashta et al., 2011; Buckley et al., 2013; Kurek et al., 2020) and is considered an edge space aerial forager. Aerial foraging bats exhibit smaller hind feet than bats foraging over water (Siemers et al., 2004). Thus, the morphometric differences between *M. davidii* and *M. mystacinus* might reflect differences in their foraging behaviour.

These morphological differences could be used to predict both the current range and the potential range expansion of *M. davidii*. Records of swarming *M. davidii* at caves in the Triglav massif in Slovenia, 25 km from our study site in Maiernigg (Çoraman et al., 2020), indicate a wider distribution of the species in the Southern Alps. As previous observations suggest water-associated foraging habitats, we hypothesize that the current distribution of *M. davidii* covers other southern Alpine water bodies like the rivers Drau, Tagliamento, Meduna and Sava as well as the lakes Ossiach, Millstatt and Bohinj. In Austria, however, our data suggest that, to date, *M. davidii* has not been documented in the comprehensive collection of the Natural History Mu-

seum of Vienna. Thus, *M. davidii* might have invaded Austria recently. The Klagenfurt basin has already been a possible entry point for other species expanding into Austria. The postglacial expansion of the Poaceae *Hierochloe australis* in Austria started in the Kanaltal and the Jauntal (eastern part of the Klagenfurt basin) (Schratt-Ehrendorfer , 1994). Findenegg (1948) published the first findings of the bat *P. kuhlii* from the Klagenfurt basin in the early 1940s, and Spitzenberger (1997) reports the first *H. savii* in Klagenfurt in 1985. Thus, our records of *M. davidii* in Klagenfurt align with those previous examples of expanding species and might indicate recent range expansion of *M. davidii*.

To further trace this range expansion, we urge bat workers to consistently measure hind foot length, tibia length and forearm length when capturing *M. cf mystacinus* or revising museum collections. For classification, we emphasise using the sex-specific LDF instead of the global LDF, because the former performed better than the latter. Moreover, to prevent incorrect determination due to erroneous LDF predictions, we recommend considering qualitative traits such as dental characteristics and colouration in putative *M. davidii* (details in Benda et al., 2012; Dietz et al., 2016. Subsequent detailed analyses of dental characteristics will confirm initial identifications based on morphometric measures and allow bat workers to identify *M. davidii* morphologically. This will help to draw a comprehensive picture of the current distribution of *M. davidii* and to understand its future range development throughout Europe.

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