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Systematics and phylogeography of the steppe whiskered bat *Myotis aurascens* Kuzyakin, 1935 (Chiroptera, Vespertilionidae)

Katerina Tsytsulina*, Matthew H. Dick, Kishio Maeda & Ryuichi Masuda

ABSTRACT. Phylogenetic relationships were examined among specimens identified as *Myotis aurascens* Kuzyakin, 1935 from across their distribution (Europe to the Korean Peninsula), and also among *M. aurascens* and other *Myotis* species. Phylogenetic reconstructions were based upon sequences of the mitochondrial cytochrome *b* and ND1 genes. In the cytochrome *b* analysis, the specimens identified as *M. aurascens* on the basis of morphology emerged as a polyphyletic group (referred to as clades A, B and C). Genetic data supported the status of clade A, which comprised most of the sequences, as a species distinct from *M. mystacinus* and the other species analysed. A paratype specimen of the form *sogdianus* Kuzyakin, 1934 appeared in the clade A of *Myotis aurascens*, which suggested clearly that they belong to the same species. However, despite that *sogdianus* Kuzyakin, 1934 should be considered a senior synonym of *aurascens* Kuzyakin, 1935, taking into consideration that a paratype does not have a name-baring function, we do not suggest to make any changes in the species name *Myotis aurascens* till further studies. In the morphometric analysis, *M. aurascens* showed a clinal pattern of variation in cranial length and most correlated measurements, which appears to be mostly independent from the mitochondrial gene patterns. *Myotis nipalensis przewalskii* appeared separately, with large genetic distances from *M. mystacinus* and the main *M. aurascens* clade. Our analysis suggests that because of the morphological similarity between *M. aurascens*, *M. nipalensis*, and the light coloured *M. mystacinus* throughout most of their distribution, identification of *M. aurascens* should be made on the basis of morphological characters, while in Europe and the Tien Shan Mountains region identifications should be made based on genetic data.

KEY WORDS: Chiroptera, Vespertilionidae, *Myotis*, *Myotis aurascens*, *Myotis mystacinus*, taxonomy, phylogeography, cytochrome *b*, ND1, mitochondrial DNA.

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Систематика и филогеография степной ночницы *Myotis aurascens* Kuzyakin, 1935 (Chiroptera, Vespertilionidae)

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РЕЗЮМЕ. На основе молекулярно-генетических данных (цитохром *b* и ND1) и морфологических данных рассмотрены взаимоотношения между экземплярами *Myotis aurascens* со всего ареала (от Европы до Корейского полуострова), а так же между *M. aurascens* и другими видами рода *Myotis*. По результатам анализа цитохрома *b*, экземпляры, определенные как *M. aurascens* на основе морфологических признаков, образовали полифилетическую группу (клады A, B и C). Молекулярно-генетические данные поддерживают самостоятельный статус клады A, в которую входит большинство рассмотренных экземпляров *M. aurascens*, как вида, отличного от *M. mystacinus* и остальных рассмотренных видов. Паратип формы *sogdianus* Kuzyakin, 1934 вошел в кладу A, что свидетельствует о том, что *sogdianus* и *aurascens* принадлежат к одному и тому же виду. Тем не менее, несмотря на то, что название *sogdianus* следовало бы считать старшим синонимом *aurascens*, в виду того, что паратип не является номенклатурным типом, мы не предлагаем изменить название вида до последующих исследований типового материала. Морфологический анализ *M. aurascens* показал наличие клинальной изменчивости в длине черепа и большинстве других скореллированных промеров, что не согласуется с географическим паттерном, основанным на молекулярно-генетических данных. В молекулярно-генетическом дереве *Myotis nipalensis przewalskii* стоит отдельно с большой генетической дистанцией как от *M. mystacinus*, так и от основной клады *M. aurascens*. На основе результатов нашего анализа, мы считаем, что из-за морфологического сходства между *M. aurascens*, *M.*

nipalensis и светлоокрашенных *M. mystacinus*, определение *M. aurascens* в центральной части ареала следует основывать на морфологических признаках, тогда как в Европе и районе Тянь-Шаня для точного определения следует привлекать генетические данные.

КЛЮЧЕВЫЕ СЛОВА: рукокрылые, Vespertilionidae, *Myotis*, *Myotis aurascens*, *Myotis mystacinus*, таксономия, филогеография, цитохром *b*, ND1, митохондриальная ДНК.

Introduction

Recently, some common and well-studied European bat species such as *Pipistrellus pipistrellus*, *Plecotus auritus* and *Myotis mystacinus* were found to consist of two or more cryptic species each (Barratt *et al*., 1997; Benda & Tsytsulina, 2000; Mayer & von Helversen, 2001b; Keifer & Veith, 2002). Though morphologically similar, these cryptic species are very different at the level of nucleotide sequences. The molecular data analysis allowed detection of inter-specific differences that were later confirmed by the morphological approach, and solved some of the problems in the taxonomy of these bats.

The *Myotis mystacinus* species complex has been studied for more than half a century (reviewed in Benda & Tsytsulina, 2000). However, even after the species status of *M. brandtii* and *M. ikonnikovi* had been shown (Hanák, 1970; Horáèek *et al*., 1974; Strelkov & Buntova, 1982; Strelkov, 1983), *M. mystacinus* sensu lato still included more than 10 subspecies.

The latest revision of the *mystacinus* group (Benda & Tsytsulina, 2000), based on morphology, substantiated the species status of *M. nipalensis*, and *M. aurascens*, and suggested that the latter species has one of the broadest geographical distributions among the group. Until recently, the eastern limit of distribution of *M. aurascens* was thought to be the Trans-Baikal region (Russia) and the Mongolian steppes. In 2003, however, two individuals, later identified by us as *M. aurascens*, were collected by K. Maeda and S.H. Han in a mountain forest in South Korea. These specimens considerably extend the known distribution of the species.

Most of the forms within *M. mystacinus* sensu lato have been described from Asia. However, as *M. mystacinus* was treated by Benda & Tsytsulina (2000) mostly from Europe, Asian forms remained problematic. Availability of scattered specimens from widely separate areas, along with morphological variation complicates a taxonomical analysis. In some cases, it is impossible to identify a particular specimen to species on the basis of morphology alone. For example, *M. mystacinus* and *M. aurascens* co-occur in Europe, where their similarity in appearance can result in misidentifications. Furthermore, in the Caucasus and Volga region, animals have been found that are intermediate in colour between the two, lighter than typical *M. mystacinus*, but darker than typical *M. aurascens* (Gazaryan, 2002; Smirnov *et al*., 2004). Then, it should be noted that even some mitochondrial DNA data have failed to support separation of *M. aurascens* from *M. mystacinus* (Mayer & von Helversen, 2001a; Ruedi *et al.,* 2002). As result, in the Third edition of the "Mammal Species of the World" *M. nipalensis* was accepted as a full species whereas *M. aurascens* was treated as synonym of *M. mystacinus* sensu stricto (Simmons, 2005). Though later some genetic evidences for *M. aurascens* species status were published (Kruskop *et al*., 2007), it is still not accepted by all the scientists.

The primary goal of the present study was to reconstruct the phylogeny of the steppe whiskered bat, *M. aurascens*, using mitochondrial cytochrome *b* (cyt *b*) and ND1 gene sequences. We also sought to determine whether genetic analyses support species status for *M. aurascens* and to examine its phylogenetic relationships with morphologically similar species (*M. mystacinus*, *M. brandtii*, *M. ikonnikovi*, and *M. muricola*). Thus we included in our analyses specimens identified as *M. aurascens* from across its range, as well as representative specimens of all other *Myotis* species available in GenBank.

Another aim of the study was to analyse geographic variation in morphological characters and phylogeographic structure within *M. aurascens* across its distributional range.

Materials and methods

Specimens included

Specimens, localities, GenBank accession numbers, and voucher depositories are listed in Appendix A. The genetic analysis included 23 specimens identified as *Myotis aurascens* from across its distributional range (Benda & Tsytsulina, 2000; Tsytsulina, 2001b); initial species identifications have been made by K. Tsytsulina, P. Benda, and S. Kruskop based on morphology. Recently, several Asian forms, originally described as subspecies of *M. mystacinus* sensu lato were preliminarily referred to *M. nipalensis* (Benda & Tsytsulina, 2000). We included sequences of the type specimen of *przewalskii* form (considered as a subspecies of *M. nipalensis*) and a paratype specimen of *sogdianus* form (a synonym of *M. nipalensis transcaspicus*). Also, we included four specimens from the Caucasus (CA135), Bulgaria (CAp2), Montenegro (CA45), and Greece (CAp1) identified as *M.* cf. *aurascens* based on qualitative characters (pelage coloration, teeth, and cranium shape). *Myotis alcathoe*, a recently described species belonging to the *mystacinus* species group, was shown to be very divergent from *M. mystacinus* sensu stricto in ND1 and 12S rRNA sequences (von Helversen *et al*., 2001). We included the ND1 sequences of *M. alcathoe*

(see Appendix A for GenBank numbers) in our analyses to examine its relationships with *M. aurascens*. Because of the recent debate about validity of *M. aurascens*, and because it was recently shown that *mystacinus* species group is not a natural unit (Ruedi & Mayer, 2001; Kawai *et al.,* 2003), we performed a combined analysis of cyt *b* and ND1 sequences, based on our own data, as well as those on *Myotis* species available from GenBank. The species names, localities and GenBank accession numbers are listed in Appendix A.

The majority of the specimens used in the study were from museum collections, and most of the samples from Asia were collected prior to the mid-1900s. The mtDNA was very fragmented in these old samples; because of this, we could not always amplify the complete cyt *b* or ND1 gene. Twenty-nine complete (1140 bp) cyt *b* gene sequences were obtained from seven *Myotis* species. We could obtain only partial cyt *b* sequences from type specimen of *przewalskii* and paratype specimen of *sogdianus* forms and eight other specimens of *M. aurascens*. Considering the importance of these samples, we included partial sequences (740 bp, from bases 1 to 400 and from 801 to 1140 bp, marked with [p] in Appendix A) of those samples into complete (1140 bp) cyt *b* set. Missing data were treated as question marks.

Eighteen complete (957 bp) ND1 sequence were obtained from specimens of seven *Myotis* species (Appendix A).

DNA extraction, amplification, and sequencing

Total genomic DNA was isolated from muscle tissue or wing membrane using a DNeasy® Tissue Kit (QIAGEN Inc), according to the manufacturer's instructions for animal tissues, except that DNA was eluted with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). For old samples, samples with possible first fixation in formalin, or when the standard procedure failed, a modified protocol (Iudica *et al*., 2001) was used. First, all tissues samples from dry skins were twice washed with distilled water, to remove naphthalene. Prolonged digestion with repeated additions of fresh 20µL aliquots of proteinase K every 12 h was carried out until the tissues were completely lysated, which sometimes took up to 96 h. On completion of the digestion process, the volume of additional proteinase K (N times × 20 µL) was calculated. The necessary volumes of AL buffer (lysis buffer; QIAGEN) and 96% ethanol were calculated for the total volume of proteinase K added. The following procedures were done according to Iudica *et al.* (2001). DNA was eluted from the QIAGEN column in 100–150 µL of TE buffer; then, depending on the extent of DNA damage, the cyt *b* gene was amplified by PCR (TaKaRa PCR thermal cycler SP) in two or more fragments, and the ND1 gene in one or two fragments. Primers used for two-part amplifications of cyt *b* were: CHCBB (5′-GAC TAA TGA CAC GAA AAA TCA CCG-3′) or L14724 (Kocher *et al*., 1989) paired with MVZ16 (Smith & Patton, 1993); L15162 (Irwin *et al*., 1991) paired with CHCBE (5′-CCT TTT CTG GTT TAC AAG ACC AG-3′) or H15915 (Irwin *et al*., 1991). Primers used for three-part amplifications of cyt *b* were: CHCBB or L14724 paired with MYC3 (5′-GTA ATT ACA GTT GCA CCT CA-3′); L15162 paired with MVZ16; MYC6 (5′-AAC TAT ATA CCA GCA AAC CC-3′) paired with CHCBE or H15195. Amplifications were done in 50 µL volumes containing from five to 10 il of the DNA extract, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 units of *Taq*DNA polimerase (TaKaRa), 0.02 mg of BSA and 0.5 mM of each primer. PCR conditions for cyt *b* were: 3 min, 94°C; 40 cycles (94°C, 1 min; 50°C, 1 min; 72°C, 1 min); 72°C, 10 min.

The ND1 gene was either amplified in its entirety using primer ER65 with ER66 (Ruedi & Mayer, 2001), or in two parts using primers ER65 with ND1r (5′-AGG AGC CAT TTA TAA GTA GAA-3′), and ND1d (5′-ACC AAT ACC ACA CCC ATT AA-3′) with ER66. PCR conditions for ND1 were: 3 min, 94°C; 40 cycles (94°C, 30 s; 52°C, 30 s; 72°C, 1.5 min); 72°C, 7 min.

In cases of degraded genomic DNA, a second round of PCR amplification with internal primers was performed under the same conditions as the first round, using 3µL from the first round as template. Sequences of the internal primers are available from the first author upon request.

PCR products were purified with a QIAquick PCR Purification Kit (QIAGEN, Chatsworth, CA) and sequenced directly with a Thermo Sequenase Core sequencing kit (Amersham, Arlington Heights, USA) and a Hitachi SQ-5500/L DNA sequencer (Hitachi Electronics Engineering Co., Tokyo).

Obtained sequences were verified and aligned using Geneworks (IntelliGenetics). As no insertions, gaps, or stop codones were present within the coding regions of cyt *b* and ND1 sequences, we assumed that all sequences were original mitochondrial genes and not their nuclear pseudogenes.

Phylogenetic analysis

To verify the position of *M. aurascens* within the genus we used all other *Myotis* species available from GenBank. The sequences obtained in the study and those from GenBank (Appendix A) were analysed in three sets: cytochrome *b* set, ND1 gene set and a combination of the two genes. Following Ruedi & Mayer (2001) *Miniopterus*, *Lasiurus*, *Scotophilus*, *Nyctalus*, *Vespertilio* and *Eptesicus* were used as complex outgroup (GenBank accession numbers are in the Appendix A). The best-fit model substitution according to Akaike information criterion (AIC) for cytochrome *b* set was TVM+I+G (rates — gamma, shape 0.6965, pinvar 0.4289), and for ND1 set and for combined set was HKY+I+G (rates — gamma, shape 0.7482 and 0.8222, pinvar 0.3364 and 0.4247, respectively) (Mod-

eltest version 3.6; Posada & Crandall, 1998). Phylogenetic analyses were undertaken using PAUP* v.4.0b10 (Swofford, 2003). Maximum parsimony (MP) trees were found using unweighted heuristic search and tree bisection–reconnection (TBR) branch swapping; 100 heuristic search replicates were performed using random addition sequence. Uninformative characters were excluded; informative characters were weighted equally and unordered. Non-parametric bootstrap values (Felsenstein, 1985) were determined by heuristic analysis of 100 pseudosamples of the original data set of informative characters, with replacement. Neighbour joining (NJ) trees were constructed using log-determinant distances. Maximum likelihood (ML) tree searches were undertaken using heuristic searches and the NJ trees were used to estimate parameters for models of nucleotide substitution and as starting trees followed by TBR branch swapping.

Mayer & von Helversen (2001a) analysed several partial (800 bp) ND1 sequences of specimens identified as *M. aurascens* and *M. mystacinus*, among other bat species from Europe. In their analysis, *M. aurascens* sequences grouped with *M. mystacinus*, with *p*-distance less than 5% within the clade. Since our data on cyt *b* and ND1 indicated that most *M. aurascens* sequences comprise a clade separate from other species, we analysed a data set that included Mayer and von Helversen's (2001a) partial sequences (GenBank numbers are in the Appendix A) as well as the our ND1 sequences.

Several measures of genetic distance have been reported in the literature for the cyt *b* and ND1 genes in bats. The most commonly used has been uncorrected *p*-distance; therefore, in order to permit comparisons, this is what we report herein. Pairwise *p*-distances were calculated with MEGA (Kumar *et al*., 1994).

Morphological analysis

Cranial and body measurements were made on 82 specimens identified as *Myotis aurascens* (specimens identified as *M.* cf. *aurascens* were not included), 28 of *Myotis mystacinus* sensu stricto, 24 of *M. ikonnikovi*, 19 of *M. brandtii*, 25 of *M. muricola* and 27 of *M. nipalensis.* It must be noted here that we use the name *M. nipalensis* following Benda & Tsytsulina (2000), though we were unable to include any information from the type specimen of this taxon. All the samples of *M. nipalensis* analysed here (both morphologically and genetically) belong to the form '*przewalskii*'. The scope of the present study does not include a comparison of these two forms. Considering the presence in Uzbekistan sample specimens belonged to very distant genetic lineages, for the morphological analysis we used only genetically identified samples and samples from the same collection series.

All specimens included in the morphological analysis (including all those tested genetically) were adults and listed in Appendix B.

External measurements included: lengths of tail, ear, tibia, foot (measured to tip of most distally extending claw), forearm, first digit (including claw), metacarpal of second digit, metacarpals and phalanges of third to fifth digits, and ear length. All wing measurements were made on the right wing.

Cranial measurements included: condylobasal length (CBL), condylocanine length (CCL), width of the skull at level of auditory bullae (W), width of braincase (BCW); height of braincase posterior to auditory bullae (BCH), interorbital constriction (IOW), rostral width at level of the preorbital foramina (WR), rostral length from preorbital foramen to alveolus of inner incisor (LR), upper tooth row length from canine to third molar (CM3), length of upper canine cingulum base (LC), width of upper canine cingulum base (WC), length of interval between cingula of upper canine and large premolar ('pseudodiastema', PD), molariform row length (P4M3), width of third upper molar (WM3), length of third upper molar (LM3), width between outer margins of upper canines (CC), width between outer margins of third upper molars (M3M3), lower jaw length from alveolus of first lower incisor to condylar process (LMD), and length of maxillary tooth row (MCM3).

For each of the morphometric characters, we evaluated the differences in mean values between species by means of analysis of variance (ANOVA); the nonparametric Kruskal-Wallis test was used because sample sizes were not equal for all species, because some measurements did not have a normal distribution, and because of the small size of some samples. All the measurements were first analysed by factor analysis, and then the 10 most substantial characters (CCL, BCW, BCH, IOW, WR, LC, WC, PD, P4M3, and M3M3) were used for the discriminant analysis and multidimensional scaling. The scaling was based on Mahalanobis distances between centroids of the groups. Significance of differences in measurements between samples and subspecies of *M. aurascens* were analysed by Mann-Whitney U Test with *p*-level not higher than 0.05 (in the most measurements <0.01). All the analyses were performed using software STATISTICA 6.0 (StatSoft Inc.). All the measurements are available from the first author upon request.

Results

Phylogenetic analyses

Cytochrome b. The aligned sequences of the complete cyt *b* gene contained 1140 characters, of which 528 were constant, 106 parsimony uninformative, and 506 parsimony informative.

In the NJ, MP and ML trees the specimens identified as *M. aurascens* based on their morphology emerge as a polyphyletic group in all the analyses (named as clades A, B, C), with the majority of the sequences united in clade A. All the cyt *b* trees consistently show two groups (B and C) as separate from the main clade A. Group B in Fig. 1 comprises two sequences: from

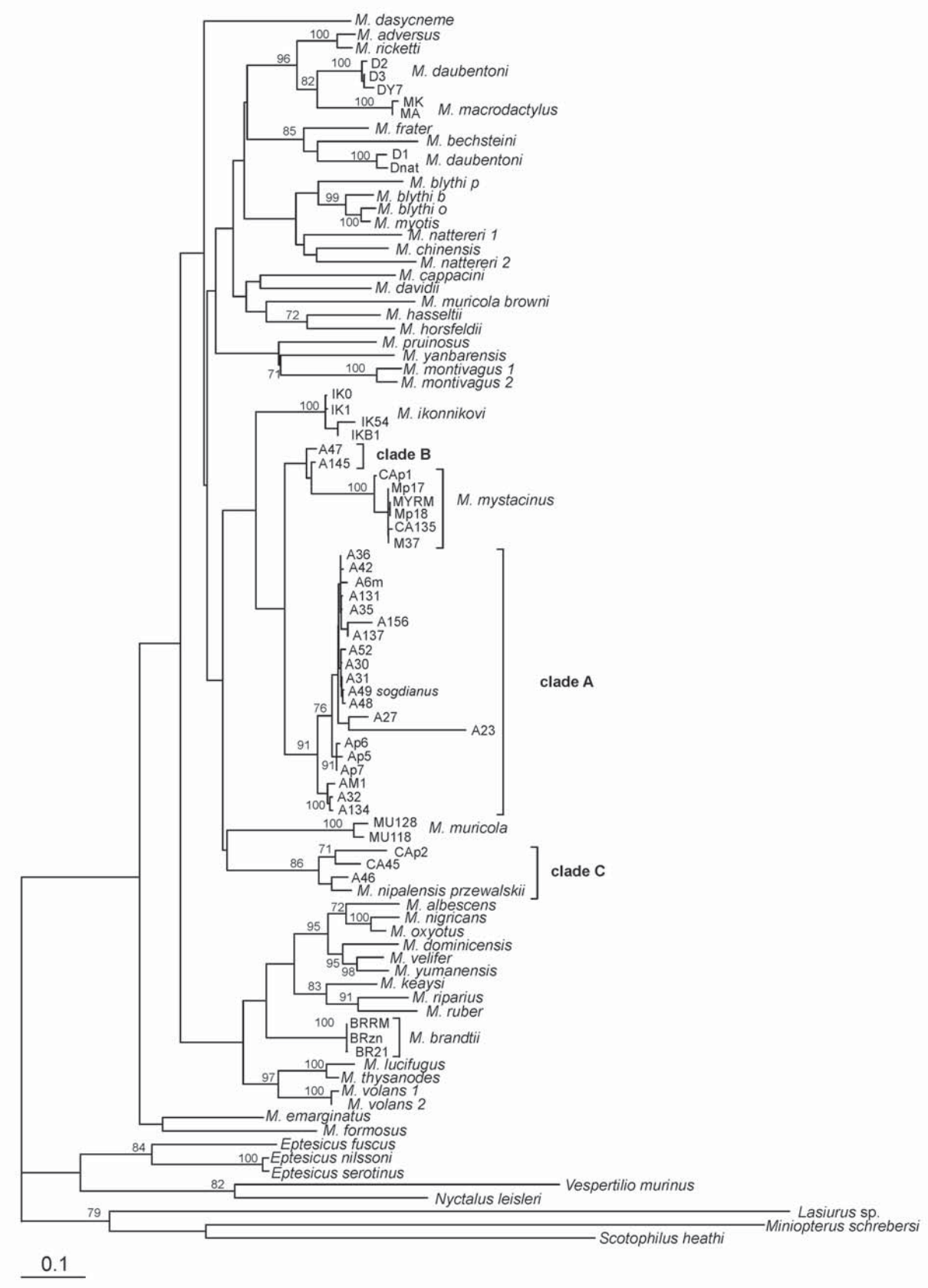

Figure 1. Maximum likelihood tree obtained for the cytochrome *b* data set (1140 bp). Numbers at the branches show bootstrap values >70% derived from MP analysis. Bootstrap values for internal branches within species are not shown.

Table 1. Uncorrected *p*-distances (above the diagonal) and ML distances (TVM+I+G; below the diagonal) for cytochrome *b* gene sequences of *M. aurascens* and 5 close related species.

cytochrome *b*	*n*	Within group		*M. aurascens*[a]	group B	clade C[b]	*M. mystacinus*	*M. ikonnikovi*	*M. brandtii*	*M. muricola*	*M. nipalensis*[c]
		ML	*p*-dist								
M. aurascens[a]	20	0.066	0.028	—	0.067	0.125	0.113	0.112	0.162	0.138	0.116
group B	2	0.022	0.022	0.088	—	0.124	0.070	0.108	0.161	0.137	0.141
clade C[b]	4	0.077	0.041	0.279	0.237	—	0.116	0.147	0.133	0.152	—
M. mystacinus	6	0.007	0.010	0.224	0.094	0.255	—	0.118	0.158	0.147	0.139
M. ikonnikovi	4	0.023	0.027	0.213	0.179	0.345	0.232	—	0.145	0.135	0.152
M. brandtii	3	0.001	0.001	0.397	0.350	0.269	0.386	0.330	—	0.160	0.133
M. muricola	2	0.031	0.034	0.317	0.276	0.392	0.355	0.297	0.476	—	0.153
M. nipalensis[c]	1	—	—	0.221	0.288	—	0.299	0.341	0.239	0.363	—

[a] under *M. aurascens* here is considered its main clade A.
[b] clade C including *M. nipalensis* sample.
[c] distances between the sample of *M. nipalensis* separately and other clades.

Mongolia (A145) and Uzbekistan (A47), which form a sister (NJ, MP) or polyphyletic (ML) group to the *M. mystacinus* clade. The average pairwise distances between them and the *M. mystacinus* clade are 7% (Tab. 1). ND1 sequences were not obtained for these two samples.

The specimens identified as *M.* cf. *aurascens* from Bulgaria (CAp2) and Montenegro (CA45) have clustered together with high bootstrap support and low genetic distance (0.9%) in all analyses (Fig. 1). Furthermore, these two sequences were consistently joining the clade composed by the specimens from Uzbekistan (A46) and *M. nipalensis przewalskii* (N50) from China (collectively, group C in Fig. 1). The average genetic distance within the group C is high, 4.1% (Tab. 1).

In all cyt *b* trees the clade A is a sister group to the *M. mystacinus* clade, which enables us to conclude on *M. mystacinus* as a monophyletic group separate from the main clade A, with all morphologically identified *M. aurascens* being polyphyletic. The bootstrap support is high for both the *M. mystacinus* clade (100%) and the clade A (91%).

Two forms considered to belong to *M. nipalensis* (*przewalskii* and *sogdianus*) appear in different clades in cyt *b* trees (Fig. 1). The sequence of *M. nipalensis przewalskii* consistently groups with the sample from Uzbekistan (A46) and *M.* cf. *aurascens* from Bulgaria (CAp2), and Montenegro (CA45). The specimen from the type series of the *sogdianus* form, recognised as a synonym of *M. nipalensis transcaspicus*, appears within the clade A. Thus, morphologically identified *M. nipalensis* emerges as a polyphyletic group.

ND1. The aligned complete ND1 sequences contained 957 characters, of which 376 were constant, 155 parsimony uninformative, and 426 parsimony informative.

The ML, MP and NJ trees based on complete ND1 sequences shows a similar topology, but is not directly comparable to the cyt *b* trees because ND1 sequences were not obtained for specimens that comprise cyt *b* clades B and C (Fig. 1). All samples identified as *M. aurascens* included in the ND1 analysis, are those which appeared in clade A of the cyt *b* set. For consistency these will also be referred to as 'clade A' when discussing the patterns from the ND1 marker, and also the combined ND1 + cyt *b* analysis. Clade A appears as a sister group to a clade containing *M. mystacinus* and *M. ikonnikovi* sequences; all of these three clades have 100% bootstrap support.

In ML, NJ, and MP trees contained partial ND1 sequences of *M. aurascens*, *M. mystacinus*, *M. brandtii* and *M. alcathoe* from Mayer & von Helversen (2001a), the homologous fragments from our data, and other *Myotis* species available in GenBank (for GenBank numbers see Appendix A) topologies of main considered branches were similar to those in complete ND1 trees (Fig. 2). Our specimens identified as *M. aurascens* on the basis of morphology (pelage coloration, cranium and teeth shape) comprise a sister clade (clade A) to a

Figure 2. Maximum likelihood tree obtained for partial ND1 data set (800 bp), combining our data with data from Mayer & von Helversen (2001a). All the sequences obtained by Mayer & von Helversen (2001a) came from Europe and are indicated by GenBank accession numbers. Numbers at the branches show bootstrap values >70% derived from MP analysis. Bootstrap values for internal branches within species are not shown.

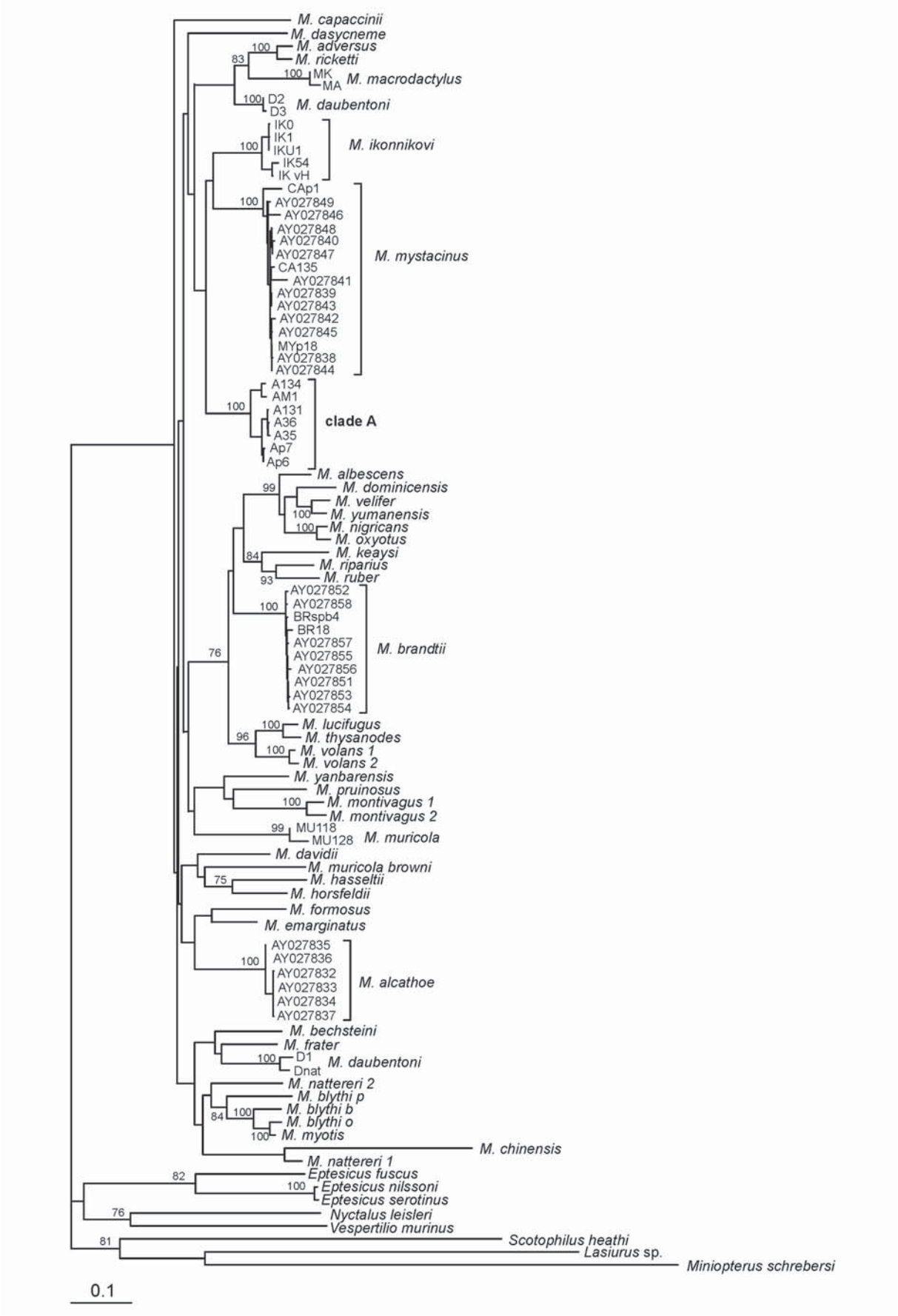
M. capaccinii
M. dasycneme
M. adversus
M. ricketti
MK
MA
M. macrodactylus
D2
D3
M. daubentoni
IK0
IK1
IKU1
IK54
IK vH
M. ikonnikovi
CAp1
AY027849
AY027846
AY027848
AY027840
AY027847
CA135
AY027841
AY027839
AY027843
AY027842
AY027845
MYp18
AY027838
AY027844
M. mystacinus
A134
AM1
A131
A36
A35
Ap7
Ap6
clade A
M. albescens
M. dominicensis
M. velifer
M. yumanensis
M. nigricans
M. oxyotus
M. keaysi
M. riparius
M. ruber
AY027852
AY027858
BRspb4
BR18
AY027857
AY027855
AY027856
AY027851
AY027853
AY027854
M. brandtii
M. lucifugus
M. thysanodes
M. volans 1
M. volans 2
M. yanbarensis
M. pruinosus
M. montivagus 1
M. montivagus 2
MU118
MU128
M. muricola
M. davidii
M. muricola browni
M. hasseltii
M. horsfeldii
M. formosus
M. emarginatus
AY027835
AY027836
AY027832
AY027833
AY027834
AY027837
M. alcathoe
M. bechsteini
M. frater
D1
Dnat
M. daubentoni
M. nattereri 2
M. blythi p
M. blythi b
M. blythi o
M. myotis
M. chinensis
M. nattereri 1
Eptesicus fuscus
Eptesicus nilssoni
Eptesicus serotinus
Nyctalus leisleri
Vespertilio murinus
Scotophilus heathi
Lasiurus sp.
Miniopterus schrebersi
0.1

Table 2. Uncorrected *p*-distances (above the diagonal) and ML distances (HKY+I+G; below the diagonal) for ND1 gene sequences of *M. aurascens* and 5 close related species.

ND1	*n*	Within group		*M. aurascens*	*M. mystacinus*	*M. ikonnikovi*	*M. brandtii*	*M. muricola*	*M. alcathoe*
		ML	*p*-dist						
M. aurascens	7	0.044	0.026	—	0.121	0.109	0.154	0.145	0.130
M. mystacinus	15	0.053	0.017	0.200	—	0.106	0.148	0.148	0.131
M. ikonnikovi	5	0.019	0.020	0.180	0.181	—	0.142	0.149	0.131
M. brandtii	10	0.014	0.006	0.302	0.285	0.276	—	0.163	0.145
M. muricola	2	0.032	0.032	0.281	0.302	0.316	0.372	—	0.153
M. alcathoe	6	0.006	0.008	0.218	0.245	0.242	0.281	0.325	—

M. mystacinus + *M. ikonnikovi* clade; bootstrap support for the clade A is 100%. In this analysis, all the ND1 sequences of *M. mystacinus* from Europe obtained by Mayer & von Helversen (2001a) clustered together with our *M. mystacinus* sequence from Europe (MYp18) and two samples of *M.* cf. *aurascens* from the Caucasus (CA135) and Greece (CAp1), with 100% bootstrap support. However, it is significant that sequences from specimens identified as *M. aurascens* by Mayer & von Helversen (these authors did not indicate which particular sequences came from specimens identified as *M. aurascens*) also appeared within the *M. mystacinus* clade. The average genetic distance within this clade is small (1.7%, Tab. 2).

Sequences of recently described *M. alcathoe* formed monophyletic clade separate from *M. mystacinus* and *M. brandtii* as it was shown by Mayer & von Helversen (2001a), as well as from the clade A.

Combined cytochrome b and ND1 set. The aligned combined set contained 2097 positions, of which 958 were constant, 219 parsimony uninformative, and 920 parsimony informative.

Clade A members of the separate analyses of cyt *b* and ND1 also appear in a monophyletic clade in all combined analyses (Fig. 3). However, in the NJ tree clade A appears to be a sister group of *M. mystacinus* + *M. ikonnikovi* clade, the same as in all ND1 trees, while in the ML and MP trees *M. ikonnikovi* is basal to clade A + *M. mystacinus* group, as in the cyt *b* trees. All three clades have high bootstrap support (100%), however the clade *M. ikonnikovi* +*M. mystacinus* + clade A is supported by relatively low bootstrap values (71%). In all trees these three morphologically and genetically close species appear together, but not necessarily with other species formerly considered as members of the subgenus *Selysius* (*M. brandtii*, *M. muricola*, *M. nigricans*, *M. dominiscensis* and *M. keyasi*). In NJ and MP their sister group is *M. muricola* from Vietnam + *M. dasycneme* (subgenus *Leuconoe*), and in ML it is *M. dasycneme* alone. Our data show that despite the genetic heterogeneity of the subgenus *Selysius*, clade A is closely related to two central species of the subgenus — *M. mystacinus* and *M. ikonnikovi*.

Average genetic distances between clade A and the other considered species range from 11.9% (*M. ikonnikovi*) to 16% (*M. brandtii*) (Tab. 3), similar to distances between *M. mystacinus* and *M. ikonnikovi,* or between *M. brandtii* and *M. mystacinus* that without any doubt are separate species. The distances between clade A and the other species are above 12% in all of the analyses (cyt *b*, ND1 and combined sets). It was shown for many vespertilionid species that this level is usually correspond to distinct species (Bradley & Baker, 2001; Ruedi & Mayer, 2001; Spitzenberger *et al*., 2001; Keifer & Veith, 2002; Kawai *et al*., 2003; Hulva *et al.,* 2004).

Phylogenetic structure of clade A

In the phylogenetic analyses of the cyt *b* and ND1 genes, most sequences from specimens identified as *M. aurascens* comprised a major clade A with high bootstrap support (91% in the cyt *b* trees, Fig. 1; 100% in the ND1 trees and in the combined cyt *b* and ND1 trees, Figs. 2, 3). In the cyt *b* trees (Fig. 1), this clade included the same 20 sequences, with A27 from Turkmenistan belonging to clade A in the NJ and ML trees, and at the base of clade A in the MP tree. Within the main clade A, three sub-clades consistently appear with high bootstrap support. One sub-clade consists of the same 13 sequences in all cyt *b* trees, but in the cyt *b* MP analysis it is lacking A27. The topology of the reduced set of this clade in the ND1 trees is consistent with that of the cyt *b* trees (Fig. 2). In the results of both cyt *b* and ND1 analyses, the second, 'western' sub-clade comprises the specimens from Iran and Crete, and the third 'eastern' sub-clade — those from Kazakhstan, Tuva, and South Korea.

The topology within clade A varies among the analyses. In the cyt *b* NJ and ML trees and the ND1 trees, the clade of samples from Iran and Crete is the sister group to the clade comprising most of the other sequences, with high bootstrap support in the ND1 trees

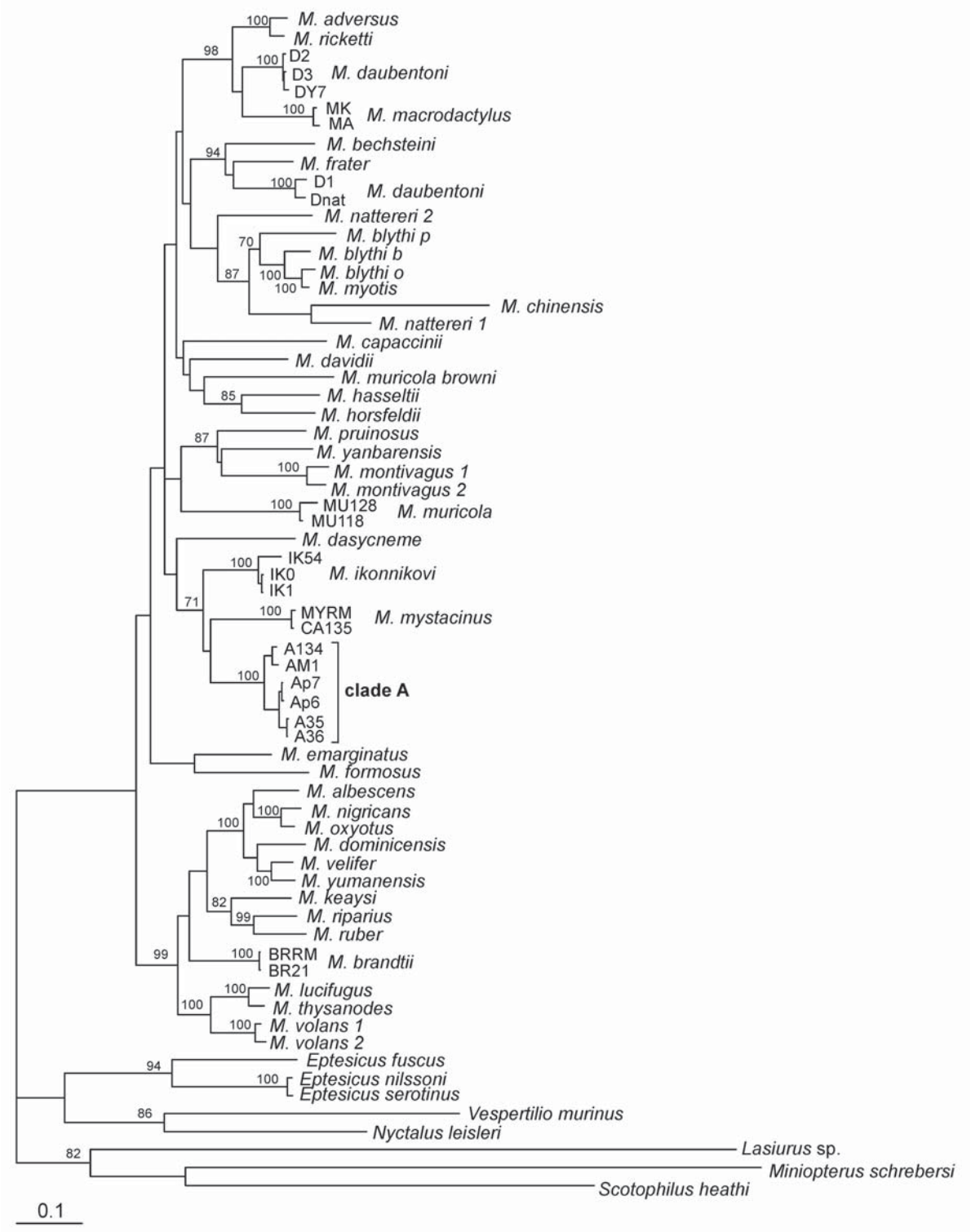

Figure 3. Maximum likelihood tree obtained for combined cytochrome *b* + ND1 data set (2097 bp). Numbers at the branches show bootstrap values >70% derived from MP analysis. Bootstrap values for internal branches within species are not shown.

(100%). In the cyt *b* MP and all of trees of combined set, two small sub-clades cluster together with high (100%, combined set) or slightly lower bootstrap support (70%, cyt *b*). Average pairwise distances between these three sub-clades for both cyt *b* and ND1 as well as for combined set are low (from 2.1% to 3.9%). Average genetic distances within the sub-clades are also low in all the sets (from 0.4% to 1.6%).

Table 3. Uncorrected *p*-distances (above the diagonal) and ML distances (HKY+I+G; below the diagonal) for combined (cyt *b* + ND1) sequences of *M. aurascens* and 4 close related species.

Combined set	*n*	Within group		*M. aurascens*	*M. mystacinus*	*M. ikonnikovi*	*M. brandtii*	*M. muricola*
		ML	*p*-dist					
M. aurascens	6	0.041	0.029	—	0.125	0.119	0.160	0.147
M. mystacinus	2	0.008	0.008	0.213	—	0.121	0.161	0.150
M. ikonnikovi	3	0.023	0.026	0.194	0.209	—	0.150	0.146
M. brandtii	2	0.002	0.003	0.349	0.349	0.313	—	0.171
M. muricola	2	0.030	0.029	0.307	0.333	0.316	0.442	—

Within clade A, mitochondrial lineages crossed geographic boundaries; that is, some sequences from geographically close localities appeared in different sub-clades. For example, among three specimens from different parts of Kazakhstan, two appeared in the main sub-clade and one in the sub-clade with samples from Tuva, and South Korea (Fig. 1). This pattern extended outside clade A as well. For example, among four specimens from Uzbekistan, two appeared in clade A (A48 and A49, the paratype of *sogdianus* from the type locality of Tashkent), one (A47) with low bootstrap support within clade B, and the remaining sequence (A46) appeared in the clade C containing *M. nipalensis przewalskii*.

Morphological analysis

The differences in the morphology and measurements between European *M. aurascens* and other species, as well as its diagnostic features, were described by Benda & Tsytsulina (2000). Here we analyse the geographic variation in *M. aurascens* across the entire distributional area of this species. We have also studied the features characteristic of *M. aurascens*, which had been proposed to differentiate the species from other closely related taxa, such as pelage coloration and teeth shape, as well as cranial and external measurements. Majority of *M. aurascens* specimens analysed, as well as *M. nipalensis*, had the 'desert type' pelage coloration, namely light fur and dark membranes, while other species examined – *M. mystacinus*, *M. brandtii*, and *M. ikonnikovi* – had the 'forest type': brown fur, and membranes – from dark brown to almost black. However, four samples treated here as *M.* cf. *aurascens* (CA45, CA135, CAp1 and CAp2 in molecular analysis) had darker pelage coloration. Three individuals, one from the Caucasus (CA135), one from Crete (CAp1) and one from Bulgaria, had fur colour intermediate between typical *M. aurascens* and *M. mystacinus*. Another specimen from the Balkans (CA45) had the colour more similar to *M. mystacinus*. Pelage coloration of *M. aurascens* from South Korea was impossible to certainly identify due to storage conditions. Nevertheless, it appears to be more similar to 'forest' type.

The shape of the canine crown is associated with length-to-width ratio and in *Selysius* species it is a stable character. Its importance for diagnostic purposes in the case of *Selysius* bats was discussed by us earlier (Benda & Tsytsulina, 2000; Tsytsulina, 2000, 2001a). *Myotis aurascens* has a rhomboid canine crown, and its length-to-width ratio is relatively uniform within the species throughout the distribution area, with exception of the specimens from Iran (*n*=4), with their thin crown and length-to-width ratio highest for the species (Fig. 4). All other examined geographical samples of *M. aurascens* did not differ somewhat significantly by the latter parameter. The Iranian sample was statistically different in this respect from the others, except the bats from Turkey and Uzbekistan. In general, *M. aurascens* differs very well by length-to-width ratio from *M. mystacinus*, *M. ikonnikovi, M. brandtii* (triangular crown with a different orientation), and *M. muricola* (polyhedral crown). *Myotis nipalensis przewalskii* (another species with rhomboid canine crown) virtually indistinguishable by length-to-width ratio from *M. aurascens,* although they have some small differences in canine shape (see Benda & Tsytsulina, 2000; Tsytsulina, 2000).

Geographical populations of *M. aurascens* show clinal variation in measurements, which is shown here on example of condylo-canine length (Fig. 5). There is a clinal trend of declining size from Europe to Uzbekistan, with reverse cline in East Asian specimens (Tuva, Trans-Baikal region, Mongolia, and South Korea). Most of the other measurements show the same pattern, though three measurements (P4M3, WM3 and LM3) vary little among all the localities. Pseudodiastema length (PD) shows a weak reverse cline, with the specimens from South Korea showing a markedly greater size than those from other localities. Also, the specimens from South Korea were the largest in overall body size among all *M. aurascens*.

Analysis of all geographical samples shows homogeneity within local populations. For example, specimens from both Kazakhstan and Uzbekistan appear in different clades in the phylogenies. However, the morphological analysis show that in both cases, the specimens from the same samples do not differ significantly

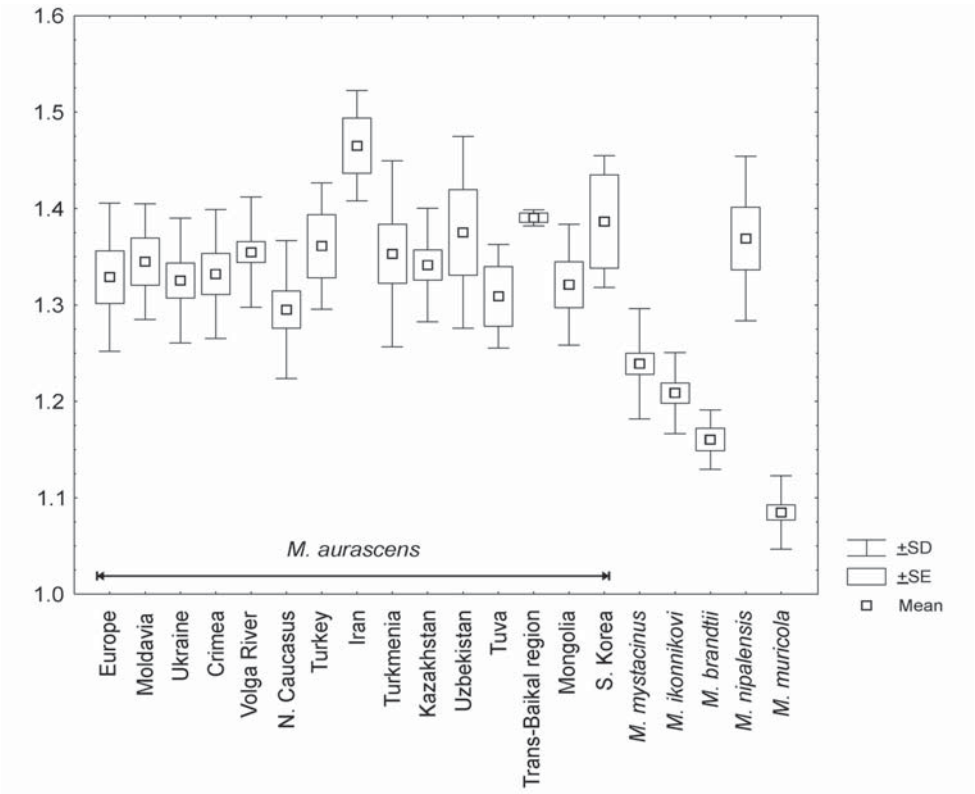

Figure 4. Canine crown length-to-width ratio in *M. aurascens* in comparison to five other species.

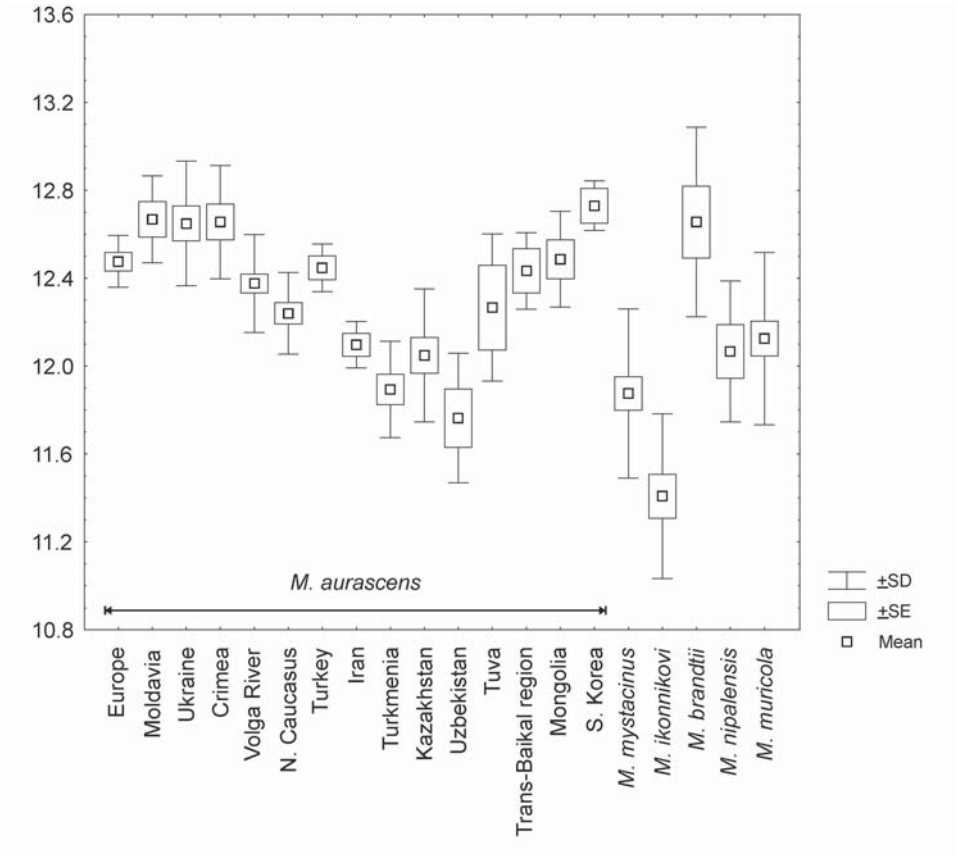

Figure 5. Geographical variation in condylo-canine length (mm) of *M. aurascens* in comparison to five other species.

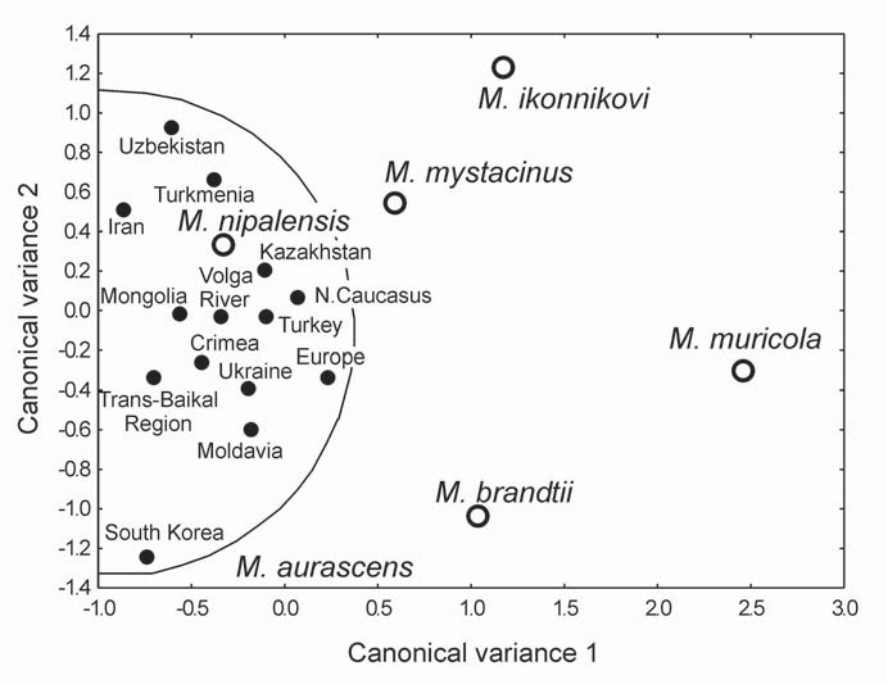

Figure 6. Multidimensional scaling based on 10 most substantial characters of *M. aurascens* geographical samples and some closely related species. The scaling was based on Mahalanobis distances between centroids of the groups.

from one another in any of the measurements analysed and belong to the same populations. Samples treated as *M.* cf. *aurascens* also belonged statistically to the corresponding geographical samples (namely Europe and the Caucasus), although they appeared on the smallest size limits. Multidimensional scaling (Fig. 6) of the specimens is in agreement with the ANOVA tests that morphologically closest species to *M. aurascens* is *M. nipalensis*. Canonical variance 1 reflects overall size, whereas canonical variance 2 reflects teeth characters (LC, WC, PD).

The shape of baculum is not very useful for distinguishing between *M. aurascens* and *M. mystacinus* or *M. nipalensis,* due to a high degree of geographic variation in its shape, as well as interspecies similarity. Nevertheless, in contrast to small baculum with weak 'wings' and head, typical of *M. mystacinus*, the one of *M. aurascens* has better pronounced lateral 'wings', usually with a border thin diaphysis, and broad epiphyses. Along with the high individual variation in bacular shape we could not find any geographic pattern. The only baculum from *M. nipalensis* we examined belongs to the subspecies *przewalskii*, which only slightly differs from that of other species by having a very narrow epiphysis.

Discussion

Taxonomic implications

Our genetic and morphological results support species status of *M. aurascens*.

Among other specimens, we analysed a paratype of a form *sogdianus* Kuzyakin, 1934, and it appeared within the main clade of *M. aurascens*. Even though *sogdianus* would be elder synonym, we do not propose to change the species name. The article 72.1.3 of the International Code of Zoological Nomenclature states that paratypes do not have a name-bearing function.

When describing *M. mystacinus sogdianus* from Uzbekistan, Kuzyakin (1934) compared the new form with *M. mystacinus transcaspicus* from Turkmenistan. The features distinguished *sogdianus* from *transcaspicus* were the position of second premolar in the lower jaw, blackish hair bases and darker membranes. Later, Kuzyakin (1935) noted that a series of samples from Uzbekistan is morphologically similar to *M. mystacinus transcaspicus*. Strelkov (1983) also did not find any significant morphological differences between forms *transcaspicus* and *sogdianus*. Both forms were preliminary assigned to the subspecies *M. nipalensis transcaspicus* by Benda & Tsytsulina (2000).

In our larger morphological set, specimens from both Uzbekistan and Turkmenistan (including genetically analysed paratype of *sogdianus* A49 and specimen from Turkmenistan A27) fit the cline of samples previously defined as *M. aurascens* (Fig. 5), and did not differ much morphologically from other clade A specimens. Even though the position of A27 is unstable in the cyt *b* trees, this specimen appeared within clade A in all the trees with high bootstrap support. Samples from Turkmenistan (A27) as well as paratype of *sogdianus*, considered to belong to *Myotis nipalensis transcaspicus* based on morphological analysis. Those two

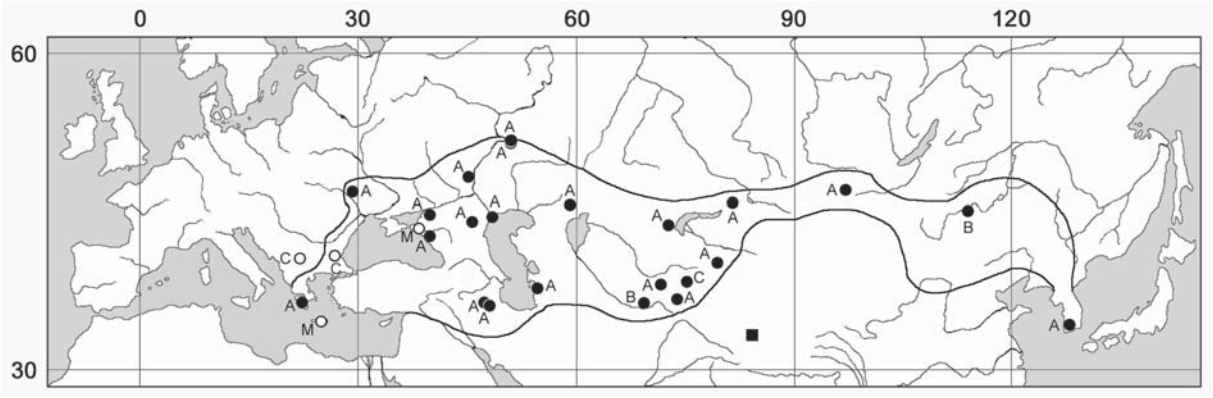

Figure 7. Approximate distribution and sampling localities of *Myotis aurascens*. Samples of *M.* cf. *aurascens* are shown with circles; sample of *M. nipalensis przewalskii* is shown with a square. Abbreviations: A — clade A; B — clade B; C — clade C; M — *M. mystacinus* clade.

samples appeared in the same clade A and thus belong to the same species, *M. aurascens*.

Benda & Tsytsulina (2000) considered *transcaspicus* to be distributed from the Caspian Sea in through Turkmenistan to Tibet Mountains. The holotype of *transcaspicus* (male, ZMMU S-29214) was described from Kopetdag Mountains, whereas analysed sample A27 originated from coastal area of Caspian Sea (Gasan-Kuli), and paratype of *sogdianus* originated from a lowland (Tashkent). It is possible that two different forms are distributed in different habitats in nearby geographical areas. A study involving more samples from different parts of the area as well as type material is necessary to establish relationships between *sogdianus*, *transcaspicus* and *aurascens*. Clarification of this question will show which name should be assigned to the species, because if all three forms belong to the same species, the eldest synonym will be *M. transcaspicus*. For now we propose to curry on with the species name *M. aurascens*, taking into consideration that we did not found any significant morphological differences between lowland, piedmont and mountainous specimens, and genetic similarity of lowland populations.

Myotis aurascens has one of the broadest distributions in the genus *Myotis*, occurring from the Europe to South Korea (Fig. 7), and similar to those of *M. brandtii*. Kruskop & Borissenko (1996) described the form *mongolicus* as a subspecies of *Myotis mystacinus* sensu lato, but subsequently we proposed that this form should be recognised as a subspecies of *M. aurascens* (Tsytsulina, 2001b) which was supported later by data came from *cox*I mitochondrial gene sequences (Kruskop *et al*., 2007). Morphological analyses indicate that form *mongolicus* is distributed eastward from Tuva, and that this form is larger in body size than specimens from other Asian localities (Fig. 5; Kruskop & Borissenko, 1996). However, our genetic analyses showed that relationships within *M. aurascens* are more complicated and do not simply reflect two geographically distinct mitochondrial sister lineages that could be interpreted as phylogenetic subspecies. Therefore, here we consider *pamirensis* Kuzyakin, 1935, *popovi* Strelkov, 1983 and *mongolius* Kruskop et Borissenko, 1996 as synonyms of *M. aurascens* Kuzyakin, 1935 without discussing their probable subspecific status.

With regard to *Myotis nipalensis*, Benda & Tsytsulina (2000), giving a new status to the form, admitted that '*nipalensis'* was highly variable and several well differentiated subspecies existed within the species. Till further study, they recognized three subspecies within *M. nipalensis*, namely *M. nipalensis nipalensis*, *M. nipalensis transcaspicus*, and *M. nipalensis przewalskii*. We were unable to examine any sample from the type series of *Myotis nipalensis*, and therefore we are not reconsidering the status of this species. Also our results did not clarify the position of *transcaspicus*. However, our results clearly show high genetic differences between the form *przewalskii* (clade C) and other *Selysius* species that supports species status of *M. nipalensis*.

Phylogenetic relationships of *M. aurascens* with other species

Our analyses of cyt *b* and ND1 genes sequences clearly differentiate *M. aurascens* along its whole distribution area from *M. mystacinus* and other *Myotis* species with long genetic distances. Four specimens regarded here as *M.* cf. *aurascens* because of their relatively dark fur coloration did not appear in clade A. Such bats have been found in Europe and the Caucasus. In the Caucasus, *M. aurascens* has a golden shimmer to the fur, whereas the light-furred form *M. mystacinus* simply has light brown fur coloration (personal observation of the first author; S. Gazaryan, personal communication). Two such samples (MYp1, Crete and MY135, the Caucasus) included in our analyses appeared within the *M. mystacinus* clade (Figs. 1, 2). Thus, we suggest that light-coloured specimens of *M. mystacinus* might have been misidentified by Mayer

& von Helversen (2001a) and Ruedi *et al*. (2002) as *M. aurascens* because of their lighter coloration and larger size than typical *M. mystacinus*. Despite the phenotype differences between *M. mystacinus* and *M.* cf. *aurascens*, the small genetic differences between them (0.9% for cyt *b* and 1.8% for ND1) allow us to conclude that they belong to the same species. The same situation has been found in the case of *Myotis lucifugus* and *M. occultus* (Piaggio *et al*., 2002). Two morphologically similar species were shown to be genetically distant by cyt *b* and cytochrome oxidase II (COII) genes' sequences, and all the morphologically intermediate specimens appeared within *M. occultus* clade. Mitochondrial genome data cannot confirm or deny hybridization; it can only be revealed by analyses of nuclear gene sequences. Therefore, Piaggio *et al*. (2002) propose that the two species have converged morphologically because of ecological factors.

Two other specimens treated herein as *M.* cf. *aurascens* (CAp2 from Bulgaria and CA45 from Montenegro), with pelage coloration more similar to *M. mystacinus*, but larger in size, consistently appeared together in all cyt *b* analyses, and were associated with two samples of *M. nipalensis*. We consider relationships between these forms unresolved until larger sample size will be analysed.

Matveev (2004) using Inter-SINE-PCR also found *M. aurascens* to be a sister group to *M. mystacinus* plus *M. ikonnikovi* clade. In our combined data set (cyt *b* and ND1 sequences) *M. mystacinus* appears to be a sister group to clade A, therefore we consider *M. mystacinus* to be the closest to *M. aurascens*. *Myotis aurascens* has a wide distribution in eastern Palaearctic, while *M. mystacinus* occurs only in Europe. Mayer & von Helversen (2001a) had shown that all European *M. mystacinus* possess similar haplotypes that form a monophyletic clade. Therefore, we speculate that *M. mystacinus* probably derived from a peripheral population of *M. aurascens*, and when they came into contact within their current distribution, they had already diverged for a long time and become reproductively isolated.

Myotis nipalensis przewalskii appeared in the group C separately from clade A (*M. aurascens*) and *M. mystacinus,* with large average distances (11.6% and 13.9%, respectively, Tab. 1). The large genetic distances indicate that *M. aurascens* and *M. nipalensis* diverged long ago, and we suggest that they have converged in morphology through adaptation to arid areas. Based on both morphological and genetic analysis we suppose that *M. aurascens* is distributed north of the Tien Shan Mountains, and *Myotis nipalensis* to the south (Fig. 7).

Distribution of *M. aurascens*

As our data show, *M. aurascens* has almost trans-Palaearctic distribution. However, it is very likely that it is not distributed further to the west than Carpathian Mountains and Greece. Further studies combining genetic and detailed morphological analyses, with larger sample sizes, need to be done in Balkan Region to determine the western limits of the distribution area. The finding of *M. aurascens* in South Korea widens appreciably the known distribution of the species; previously the easternmost record was from the eastern Mongolia. In South Korea, *M. aurascens* was found in a summer roost located in secondary broadleaf mountain forest. This habitat is similar to that of *M. aurascens* in Europe and the Caucasus. In the western part of its range the species is found in cracks in rock outcrops and chinks under bridges in the summer and in caves and old mines during hibernation (K. Tsytsulina, personal observations; Ilyin *et al*., 1998; Gazaryan, 2002). In South Korea *M. aurascens* was also found in summer in cave cracks near exits. Gazaryan (2002) found *M. aurascens* in the Caucasus during hibernation in mountain caves less than 500 m above sea level, with temperature above 8ºC, and did not find it higher in the mountains, where the temperature in caves is lower. In Europe and Korea *M. aurascens* inhabits broad-leaf, mixed or subtropical forests, and in the middle part of the distribution it inhabits steppes and semi-deserts and does not occur in forests to the north. All the records of *M. aurascens* came from areas with relatively high ambient temperature, in contrast to *M. mystacinus* and *M. brandtii* that are found as far as the Ural Mountains in the north. Therefore, in our opinion, the distributional pattern of *M. aurascens* is related much more to higher ambient temperatures than to landscape.

Phylogeographic structure of *M. aurascens*

Myotis aurascens shows clinal variation in morphology that is not well correlated with the pattern from mitochondrial genes. Over the main part of its distribution (Europe to Uzbekistan), *M. aurascens* demonstrates a decrease in size from west to east. Samples of *M. aurascens* from the eastern part of the range increase in size from north-west to south-east. However, despite the presence of these two morphological clines, molecular evidence suggested low genetic variation within the species (clade A). A similar pattern, with a morphological cline combined with low sequence divergence within the species, was observed in *Rhinolophus cornutus* and *Rh. ferrumequinum* (Yoshiyuki, 1989; Sakai *et al*., 2003). The lack of strong phylogeographic structure has been found previously in other highly mobile animals with low population densities such as wolves (Vila *et al*., 1999), jackals (Wayne *et al*., 1990) or bears (Hofreiter *et al*., 2004). However, both cyt *b* and ND1 genes have a similar relative slow rate of evolution in bats (Ruedi & Mayer, 2001), making them suitable for studies of species level relationships and broad phylogeographic patterns (Avise, 2000). Inter-population relationships are best studied with alternative markers such as D-loop or microsatellites.

The combination of both molecular (cyt *b* and ND1 genes sequences) and paleontological data suggests that the differentiation of most Palaearctic and Oriental

species of *Myotis* took place during the Miocene, roughly between 5 and 9 Mya (mean 6.5 ± 1.6 Mya) (Ruedi & Mayer, 2001). The divergence rates from the present study suggest that clades comprising morphologically similar bats (clades A = *M. aurascens*, B and C, which includes *M. nipalensis*) diverged long ago and reached similarity in morphology due to adaptation to a similar environment. We suggest that after the glacial period, already being diverged genetically, but probably not very much morphologically, considered forms radiated to the north and occupied the arid steppe, semi-desert and mountain sub-alpine zones. Bats from different refuges in some cases came to inhabit the same region (e.g. Uzbekistan), where they acquired uniformity in morphology due to adaptation to the same local selective pressures.

As the morphological differences between *M. aurascens*, *M. nipalensis przewalskii* and light coloured *M. mystacinus* are very small (though not absent), identification of *M. aurascens* throughout most of its distribution could be made based on morphological characters only, while in Europe and the Tien Shan Mountains region identifications should be made with the use of examination of mtDNA sequences.

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Appendix A. Specimens included in the bat mtDNA analyses.

Species	DNA sample number	GenBank accession numbers		Locality	Voucher[1]
		cytochrome *b*	ND1		
M. adversus		**AB106587**	**AB106566**		Kawai 2003
M. albescens		**AF376839**	**AY033952** [p]		RM 2001
M. alcathoe			**AY027832** [p]		vH 2001
			AY027833 [p]		vH 2001
			AY027834 [p]		vH 2001
			AY027835 [p]		vH 2001
			AY027836 [p]		vH 2001
			AY027837 [p]		vH 2001
Myotis aurascens	A6m	**AY665155**	—	Russia, Rostov-on-Don	ZMMU 171259
	A23	**AY699836** [p] **AY699846**	—	Russia, The Caspian Sea, Astrakhan	ZIN 5721
	A27	**AY665160**	—	Turkmenia, Gasan-Kuli	ZIN 56940
	A30	**AY665158**	—	Eastern Kazakhstan, Ayagoz	ZIN 68541
	A31	**AY665157**	—	Kyrgyzia, Issuk-kul' Lake	ZIN 65742
	A32	**AY665146**	—	Central Kazakhstan, west of Balhash Lake	ZIN 68247
	A35	**AY665151**	**AY699856**	Russia, North Caucasus, Stavropol Region	ZIN 78226
	A36	**AY665154**	**AY699857**	Russia, Volgograd Region	ZIN 78228
	A42	**AY665153**	—	Moldavia	ZIN 62425
	A46	**AY699837** [p] **AY699847**	—	Uzbekistan, Fergana	ZIN 57302
	A47	**AY699838** [p] **AY699848**	—	Uzbekistan, Termez	ZIN 41678
	A48	**AY699839** [p] **AY699849**	—	Uzbekistan, Gissarsky Mt. ridge	ZIN 24392
	A49 *sogdianus*	**AY699840** [p] **AY699850**	—	Uzbekistan, Tashkent	ZIN 41677
	A52	**AY665159**	—	Western Kazakhstan, Emba River	ZIN 65110
	A131	**AY665152**	**AY699858**	Russia, North Caucasus, Tuapse	ZMMU 166220
	A134	**AY665147**	**AY699859**	Russia, Tuva	ZMMU 168601
	A137	**AY665156**	—	Russia, Middle Volga River, Engels	ZMMU 160793
	A145	**AY699841** [p] **AY699851**	—	Mongolia, Choybalsan	ZMMU 130416
	A156	**DQ182698**	—	Russia, Middle Volga River, Engels	ZMMU 160787
	AM1	**AY665148**	**AY699860**	South Korea, Gangwon Do, Yongwol Gun	KM 13093
	Ap5	**AY699842** [p] **AY699852**	—	Greece, Messina	NMP 51477
	Ap6	**AY665150**	**AY699861**	Iran, Western Azaerbaidjan	NMP 48120
	Ap7	**AY665149**	**AY699862**	Iran, Western Azaerbaidjan	NMP 48119
M. cf. *aurascens*	CA45	**AY665142**	—	Montenegro	ZIN 35064
	CA135	**AY665141**	**AY699863**	Russia, Caucasus, Gelendjik	ZMMU 171262
	CAp1	**AY699843** [p] **AY699853**	**AY699864**	Greece, Crete	NMP 48345
	CAp2	**AY665164**	—	Bulgaria	NMP 48342
M. bechsteini		**AF376843**	**AY033978** [p]		RM 2001
M. blythii blythii	*M. blythii b*	**AF376840**	**AY033966** [p]		RM 2001
M. blythii oxygnathus	*M. blythii o*	**AF376841**	**AY033988** [p]		RM 2001
M. blythii punicus	*M. blythii p*	**AF376842**	**AY033959** [p]		RM 2001
M. brandtii	BR18	—	**AY699868**	Russia, Ural Mountains	ZIN 82785
	BR21	**AY665139**	—	Russia, Moscow Region	ZIN 82729
	BRzn	**AY665168**	—	Czech Republic, Znojmo	—
	BRspb4	—	**AY699869**	Russia, Leningrad Region	NMP 49272
	BRRM	**AF376844**			RM 2001
			AY027851 [p]		vH 2001
			AY027852 [p]		vH 2001
			AY027853 [p]		vH 2001
			AY027854 [p]		vH 2001
			AY027855 [p]		vH 2001
			AY027856 [p]		vH 2001
			AY027857 [p]		vH 2001
			AY027858 [p]		vH 2001

Appendix A (continued).

Species	DNA sample number	GenBank accession numbers		Locality	Voucher[1]
		cytochrome *b*	ND1		
M. capaccinii		**AF376845**	**AY033989** [p]		RM 2001
M. chinensis		**AB106588**	**AB106567**		Kawai 2003
M. dasycneme		**AF376846**	**AY033977**		RM 2001
M. daubentoni	Y7	**AY665137**	**AY699872**	Japan, Hokkaido	KT Y7
	D1	**AF376847**	**AY033985** [p]		RM 2001
	D2	**AB106589**	**AB106568**		Kawai 2003
	D3	**AB106590**	**AB079824**		Kawai 2003
	Dnat	**AF376862**	**AY033954** [p]		RM 2001
M. davidii		**AB106591**	**AB106569**		Kawai 2003
M. dominicensis		**AF374868**	**AY033965**		RM 2001
M. emarginatus		**AF376849**	**AY027859** [p]		RM 2001, vH 2001
M. formosus		**AB106592**	**AB106570**		Kawai 2003
M. frater		**AB106593**	**AB106571**		Kawai 2003
M. hasseltii		**AF376850**	**AY033973** [p]		RM 2001
M. horsfeldii		**AF376851**	**AY033970** [p]		RM 2001
M. ikonnikovi	IK54	**AY665162**	**AY699866**	Russia, Irkutsk Region	ZIN 83809
	IKB1	**AY665165**	—	Russia, Sakhalin	BM 39061
	IKU1	—	**AY699867**	Japan, Hokkaido	—
	IK0	**AB106594**	**AB106572**		Kawai 2003
	IK1	**AB106595**	**AB106573**		Kawai 2003
	IK2	**AB106596**	**AB106574**		Kawai 2003
	IKvH		**AY027850**		vH 2001
M. keaysi		**AF376852**	**AY033963** [p]		RM 2001
M. lucifugus		**AF376854**	**AY033967** [p]		RM 2001
M. macrodactylus	MA	**AY665163**	**AY699873**	Japan, Honshu	KT J5
	MK	**AB106604**	**AB106582**		Kawai 2003
M. montivagus	1	**AF376857**	**AY033972** [p]		RM 2001
	2	**AF376858**	**AY033971** [p]		RM 2001
M. myotis		**AF376860**	**AY033986** [p]		RM 2001
M. mystacinus	MYp17	**AY665167**	—	Slovakia, Rovne	NMP 49500
	MYp18	**AY665140**	**AY699865**	Czech Republic, Znojmo	NMP 49495
	MY37	**AY665166**	—	Russia, Ural Mountains	ZIN 82786
	MYRM	**AF376861**			RM2001
			AY027838 [p]		vH 2001
			AY027839 [p]		vH 2001
			AY027840 [p]		vH 2001
			AY027841 [p]		vH 2001
			AY027842 [p]		vH 2001
			AY027843 [p]		vH 2001
			AY027844 [p]		vH 2001
			AY027845 [p]		vH 2001
			AY027846 [p]		vH 2001
			AY027847 [p]		vH 2001
			AY027848 [p]		vH 2001
			AY027849 [p]		vH 2001
M. muricola	MU118	**AY665144**	**AY699870**	Vietnam, Cat Tien	ZMMU 172613
	MU128	**AY665143**	**AY699871**	Vietnam, Lo Go Xa Mat	ZMMU 172623
M. muricola browni		**AF376859**	**AY033958** [p]		RM 2001
M. nattereri	*1*	**AF376863**	**AY033984** [p]		RM 2001
	2	**AB106606**	**AB106584**		Kawai 2003
M. nigricans		**AF376864**	**AY033983** [p]		RM 2001
M. nipalensis przewalskii	N50	**AY699844** [p] **AY699854**	—	China, Uygur Autonomous Region (former Kashgaria)	ZIN 13903
M. oxyotus		**AF376865**	**AY033956** [p]		RM 2001
M. pruinosus		**AB106607**	**AB106585**		Kawai 2003
M. ricketti		**AB106608**	**AB106586**		Kawai 2003
M. riparius		**AF376866**	**AY033982** [p]		RM 2001
M. rubber		**AF376867**	**AY033981** [p]		RM 2001
M. thysanodes		**AF376869**	**AY033957** [p]		RM 2001
M. velifer		**AF376870**	**AY033980** [p]		RM 2001
M. volans	*1*	**AF376871**	**AY033960** [p]		RM 2001
	2	**AF376872**	**AY033961** [p]		RM 2001

Appendix A (continued).

Species	DNA sample number	GenBank accession numbers		Locality	Voucher[1]
		cytochrome *b*	ND1		
M. yanbarensis		**AB106610**	**AB079828**		Kawai 2003
M. yumanensis		**AF376875**	**AY033979** [p]		RM 2001
Eptesicus fuscus		**AF376835**	**AY033968** [p]		RM 2001
Eptesicus nilssoni		**AF376836**	**AY033987** [p]		RM 2001
Eptesicus serotinus		**AF376837**	**AY033950** [p]		RM 2001
Lasiurus sp.		**AF376838**	**AY033975** [p]		RM 2001
Miniopterus schreibersii		**AF376830**	**AY033969** [p]		RM 2001
Nyctalus leisleri		**AF376832** [p]	**AY033949** [p]		RM 2001
Scotophilus heathi		**AF376831**	**AY033974** [p]		RM 2001
Vespertilio murinus		**AF376834**	**AY033964** [p]		RM 2001

[p] — partial sequences. In the case of cyt *b*, 740 bp, first accession number corresponds to 1-400 bp of cytochrome *b* gene, second accession number corresponds to 801–1140 bp. In the case of ND1 partial sequences were obtained from GenBank and in majority correspond to 1–800 bp of the ND1 gene.

1. Voucher acronyms: BM — Burke Museum of Natural History and Culture, University of Washington, USA; NMP — National Museum of Prague, Prague, Czech Republic; ZMMU — Zoological Museum of Moscow University, Moscow, Russia; ZIN — Zoological Institute of Russian Academy of Science, Saint Petersburg, Russia; KT — private collection of K. Tsytsulina; Kawai 2003 — all details on location and voucher are published in Kawai *et al*., 2003; RM 2001 — all details on location and voucher are published in Ruedi & Mayer, 2001; vH 2001 — all details on location and voucher are published in von Helversen *et al*., 2001.

Appendix B

The list of examined *Myotis* specimens (species, region, number and sex, collection numbers). Collections acronyms are as follows: BM — Burke Museum of Natural History and Culture, University of Washington, USA; BPI — Institute of Biology and Soil Science, Vladivostok, Russia; ISU — Zoological Museum of Irkutsk State Univerity (Irkutsk, Russia); KM — Nara Educational University, Nara, Japan; MUU — Museum of Uppsala University (Uppsala, Norway); NHMS — National Natural History Museum (Bulgaria, Sofia); NMP — National Museum (Natural History) (Prague, Czech Republic); NSMT — National Science Museum in Tokyo, Japan; PSEU — Penza State Educational University (Penza, Russia); ZMMU — Zoological Museum of Moscow University, Moscow, Russia; ZIN — Zoological Institute of Russian Academy of Sciences, Saint-Petersburg.

M. transcaspicus — Europe (22 ♀♀, 15 ♂♂, 3u): ZIN 32630, 35062, 38778, 41665–66, 49554–55, 53032–38, 55594–96, 55692–93, 62424–26, 62691, 78725; ZMMU 4158–59, 5022, 5024–25, 29157, 29432, 84006–08; NMP PMS 6688, ZMS 11, 92, 94, 117, 139; Caucasus and Crimea (30 ♀♀, 21 ♂♂, 8u): ZIN 4909–10, 5156, 6031, 8057, 8182, 9014, 9019, 9178, 9189, 9249–51, 23506–07, 45249, 68508–09, 69879, 77520, 78226, 78274–77, 78288, 80877, 80848–54, 81824, 76-1916; ZMMU 9266–67, 40903–05, 29243–44, 29432, 32496, 46560–65, 40903–05, 84003, 104469–70, 160561, 166219–20; Iran (4u): ZIN 5118, 5154, NMP 48119–20; Middle Asia, Kirgizia and Kazakhstan (68 ♀♀, 1 ♂): ZIN 56675–82, 62172–81, 65107–11, 68322–32, 68334–47, 68515–25, 69170, 62172–81; Trans-Baikal region, Tuva and Mongolia (30 ♀♀, 21 ♂♂, 8u): ZIN 5238, 20205–07, 26690, 49756–57, 53936–40, 58539–44, 58458–62, 66400; ZMMU 42918, 49920, 49922–25, 130415–17, 148474–76; South Korea (1 ♀, 1 ♂): KM 12253, 13093.

M. nipalensis przewalskii — Middle Asia (10 ♀♀, 4 ♂♂, 13u): ZIN 2147, 5118, 5154, 5327, 83983, 13903–10, 23140, 23147, 31857, 41677, 41679, 56674, 57301, 57302, 84954, 98-1914; ZMMU 6819, 9265, 29228, 104443; China (5u): ZIN 2147, 13904–07.

M. mystacinus — Europe (5 ♀♀, 17 ♂♂, 7u): MUU A534619, A530064-65, A534407, A590186; ZIN 35062, 35064–66, 62630, 82623–28; ZMMU 74666; NMP GR-1, TH-5, TH-16, 48344–46, 51477, PMS 5329, PMS 5553, C-42; NHMS ZMS 95, ZMS 138; European part of Russia, Byelorussia (5 ♀♀, 2 ♂♂): ZIN 5121, 70669; ZMMU 54823, 154732–33, 164726, 162552; Volga River Basin (6 ♀♀, 6 ♂♂): ZIN 37899, 37919, 72522–31; Ural Mountains (6 ♀♀, 11 ♂♂, 3u): ZIN 23484, 37899–900, 37910–13, 37915–16, 37918–19, 37979, 41681, 41684, 82784, 82786; PSEU 915p, 913p, 912p; ZMMU 104463; Caucasus (11 ♀♀, 13 ♂♂, 11u): ZIN 5345–48, 8929, 9001–04, 9006, 9008–13, 9018, 9177, 9286–87, 9582, 23170, 23506, 49743, 69582, 82432, 83008, 83623, 83771, 83674–75; ZMMU 4168a, 21536, 32496, 104471.

M. brandtii — Europe (24 ♀♀, 22 ♂♂, 5u): ZIN 9192, 9206, 35063, 23482–83, 29489, 33468, 36817, 38545–46, 38548, 38796, 40690–92, 40721, 40727, 40851–52, 41669, 42909, 42821, 43140, 55709–10, 62429, 69044–48, 82729, 82730, 84006, 84713–14; ZMMU 74665, 154728–33; NMP B-0476, B-0733; ZMS 11, 94, 97, 120, 139; MUU À530063; Caucasus (4 ♀♀, 4 ♂♂): ZIN 9253, 9260, 23490, 78286–87, 80876; Ural Mountains and Volga River Basin (12 ♀♀, 17 ♂♂, 9u): ZIN 5080, 5153, 5919, 33614, 37899, 37900–03, 37907–09, 37914, 41664, 41681–87, 69041–43, 70704–705, 72369, 82465; ZMMU 4165, 83899, 104458–62, 104464, 162551, 164191; Siberia (16 ♀♀, 16 ♂♂, 1u): ZIN 59614–33, ZMMU 65925, 104434, 104435; Trans-Baikal region (8 ♀♀, 4 ♂♂, 3u): ZIN 49755, 66109–10, 66112–13, 66116–19, 66121–23, 66126,

66127, ISU 352; Russian Far East (7 ♀♀, 10 ♂♂, 8u): ZIN 4722–24, 4733, 9844, 39437–38, 40404–05, 41671–72, 41675, 84013; ZMMU 40504, 51175, 104422–27; BPI 527, 557, 569–570; Kamchatka (1 ♀, 2 ♂♂): ZMMU 51175, ZIN 24-1913(1), 49759; Sakhalin (1 ♀, 1 ♂): ZIN 41671, ZMMU 35351; Hokkaido, Japan (38 ♀♀, 11 ♂, 5u): NSMT 15194, 46; KM 546–52, 554–61, 2843–44, 3049, 3051, 3058–60, 3063, 3070, 3119, 312-24, 3126, 3152–53, 11299–301, 12199, 12202, 12206–08, 12225, 12228–32, 12316–19, 12336.

M. ikonnikovi — Altai Mountains (3 ♂♂, 2u): ZIN 75606, 83984; ZMMU 28576–77, 33157; Eastern Siberia (6 ♀♀, 3 ♂♂, 5u): ZIN 5101, 5375, 8997, 63809, 66505, 77172, 77632, 83809, 85535; ZMMU 65975; NMP 60, ESN227/18, ESN884/39, AR93/108; Mongolia (2 ♂♂, 2u): ZIN 5190, 13914, Russian Far East (18 ♀♀, 13 ♂♂, 5u): ZIN 5127, 8997, 9254, 30451, 49998–50000, 62310, 63809, 81699, 81701; ZMMU 50954, 52493, 84009, 96372, 103913, 104418–21, 110031, 158583, 165490–92, 165522; BPI 815, 958, 976, 984, 1001, 1004–05, 1007, 1043, 53-89; Kamchatka (1 ♀): BM 39061; Sakhalin (1u): ZIN 62310; Hokkaido, Japan (17 ♀♀, 22 ♂♂): KM 665, 2795, 2800–04, 2839, 3122, 3125, 3149–51, 3154, 3156–57, 6745, 6748, 12210–11, 12348–49, 12433–40, 12442–43, 12445, 12488, 12837, 12842, 12890, 12904–06, 12943, 12986.

M. muricola — Nepal (2 ♂♂): ZMMU 164491–92; Vietnam (3 ♀♀, 4 ♂♂): ZIN 5508, 5510–5512, ZMMU 165048, 165055, 167188; Cambodia (2 ♀♀, 4 ♂♂): ZMMU 166163–64, 168335–38; China (2 ♂♂): ZIN 5929–30; Sumatra (5 ♂♂): ZIN 84715–19; Java (15u): ZMMU 103257–71.