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Variations on a theme: Karyotype comparison in Eurasian *Myotis* species and implications for phylogeny

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Abstract. The phylogenetic relationships within the large genus *Myotis* are still unsolved to a high degree although several morphological and molecular studies have been undertaken recently. In cytogenetic respect, *Myotis* is a very homogeneous genus as nearly all species show a karyotype with 44 chromosomes. Application of banding techniques in 17 Eurasian *Myotis* taxa revealed complete conservation of chromosomes. However, variation has been found concerning position and extent of heterochromatic segments and location and number of nucleolus organizer regions (NORs). In three cases these differences may be of relevance for phylogenetic relationships. The close affinity of *M. myotis*, *M. blythii* and *M. nattereri* shown in molecular studies was confirmed by a common interstitial heterochromatic segment on chromosome 15 in these species. The presence of two cryptic species in *M. montivagus*, suspected earlier for the reason of a 5% difference in mtDNA sequences, was fortified by the observation of a distinct karyological difference, i.e. a pericentric inversion on chromosome 7. In contrast to previous molecular studies, Greek specimens of *M. mystacinus* were clearly differentiated from German specimens concerning the location of NORs and size and morphology of the Y chromosome. The Greek specimens were provisionally assigned to the subspecies *M. mystacinus bulgaricus*.

Chromosome, heterochromatin, NOR, G-banding

Introduction

The world-wide distributed genus *Myotis* Kaup, 1829 comprises about 100 species (Simmons 2005). It probably has the widest natural distribution of any genus of terrestrial mammals except *Homo* (Nowak 1994). Examination of morphological characters in the genus *Myotis* resulted in identification of up to seven subgenera (Tate, 1941, see Fig. 1). Findley (1972), however, recognized only three subgenera (*Myotis*, *Leuconoe* and *Selysius*), which lately were reduced to two by Menu (1987) and Godawa Stormark (1998) by including the members of *Selysius* into *Leuconoe*. The first molecular systematics of the genus based on the cyt *b* and nd1 genes (Ruedi & Mayer 2001) revealed a division of *Myotis* into 5 clades. From these results, Ruedi & Mayer (2001) concluded that independent radiations produced strikingly similar evolutionary solutions in different parts of the world. Concerning European species of *Myotis*, the most surprising result of this study was the affiliation of *M. brandtii* to the American clade. Therefore the authors suspected that this species has colonized Europe secondarily probably through the Beringean Bridge (Ruedi & Mayer 2001). Several papers dealing with the molecular phylogeny of a great variety of *Myotis* species have been published in the meantime (Kawai et al. 2003, Bickham et al. 2004, Stadelmann et al. 2004, 2007, Zhang et al. 2009, Jiang et al. 2010, Tsytsulina et al. 2012), mostly based on cyt *b* alone, sometimes also on nd1 or another mitochondrial gene, cytochrome oxidase subunit 1 (Kruskop et al. 2012). The general picture of all of these studies is the separation of the genus into several clades, from 5 as in Ruedi & Mayer (2001) and Stadelmann et al. (2004, 2007), to 7 (Zhang et

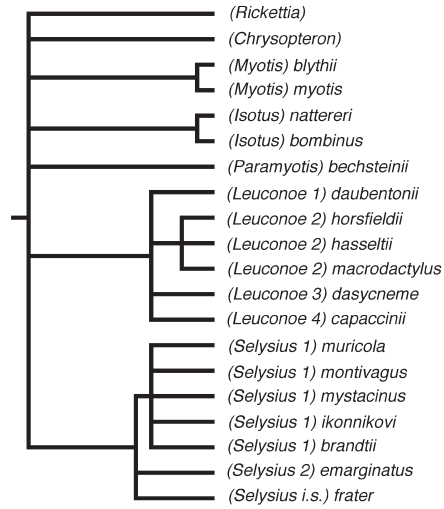

Fig. 1. Cladogram constructed after Tate's (1941) arrangement of the Eurasian species and named forms of *Myotis* under subgenera. Subgeneric names are given in brackets. Only those species for which sufficient chromosomal data exist are shown. For clarity, the names of Tate's sections have been replaced by numbers as follows: *Leuconoe* 1: *daubentonii*; *Leuconoe* 2: *adversus*; *Leuconoe* 3: *dasycneme*; *Leuconoe* 4: *capaccinii*; *Selysius* 1: *mystacinus*; *Selysius* 2: *emarginatus*; *Selysius* i.s.: incertae sedis.

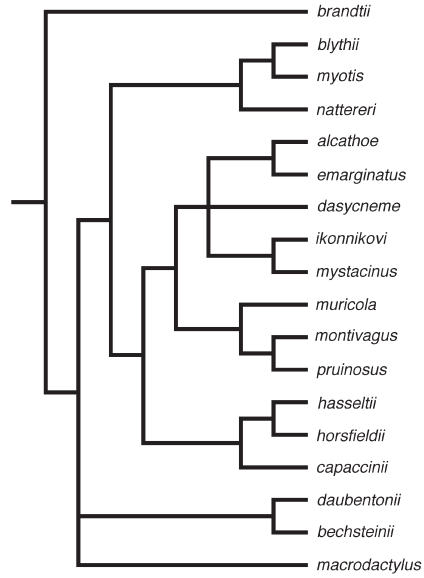

Fig. 2. Phylogenetic relationships of *Myotis* species based on cytochrome *b* sequence analysis (Stadelmann et al. 2007; Fig 2a, modified). Only Eurasian species for which sufficient chromosomal data exist are shown here. The length of the branches has been chosen arbitrarily.

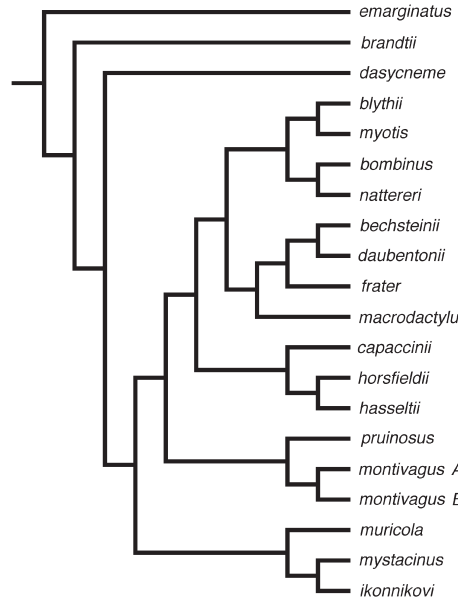

Fig. 3. Phylogeny of karyologically studied Eurasian *Myotis* species based on cytochrome *b* sequence analysis (modified after Tsytsulina et al. 2012; Fig. 1, maximum likelihood tree). The length of the branches has been chosen arbitrarily. The *M. montivagus* specimens shown here are identical with those studied karyologically.

al. 2009) or even 9 (Kawai et al. 2003). However, for some species (e.g. *Myotis emarginatus*, *M. dasycneme*) the affiliation to a certain clade varies from study to study. And, more important, the phylogenetic relations between the clades could not be solved due to the low bootstrap support of the nodes with the present data. This problem might be a reflection of a rapid species diversification (Ruedi & Mayer 2001). To demonstrate the contrasting molecular results, we selected two examples from the papers cited above, which included most of the karyologically studied Eurasian species. We removed those species without sufficient chromosomal data from the published trees. These modified phylogenies are shown in Fig. 2 (Stadelmann et al. 2007) and Fig. 3 (Tsytsulina et al. 2012).

From a cytogenetic point of view, the genus *Myotis* is a very homogeneous taxon with the overwhelming majority of species showing a karyotype with a diploid chromosome number of 44. A careful examination and application of different banding techniques showed, however, that indeed small differences can be found in quite a number of species.

In the present paper we describe the karyotypes of 17 *Myotis* taxa from Europe and South East Asia and compare them with published data of other Eurasian species.

Material and Methods

Cell culture, metaphase preparation and chromosome staining methods have been described elsewhere (Volleth 1987, Volleth et al. 2009). Chromosomal arms were numbered using Bickham's scheme for American *Myotis* species (Bickham 1979).

Table 1. Specimens examined. Data for additional specimens can be found in Volleth (1987). Abbreviations: ID identification number of specimen; SMF – Senckenberg Museum Frankfurt; B – Bavaria; Dr – Drama; E Spain; Ft – Fthiotis; GR – Greece; G – Germany, K – Karditsa; MAL – Malaysia; R – Russia; Th – Thessaloniki

species	code	ID	sex	locality	SMF number
M. alcathoe[1]	MAL	363	F	GR, K: Loutropigi	
	MAL	366	F	GR, K: Loutropigi	
	MAL	367	F	GR, K: Loutropigi	
M. blythii	MBL	305	M	GR, Ft: Pavliani	87497
M. daubentonii	MDA	348	M	G, B: Dechsendorf	
	MDA	375	M	E: El Burgo de Osma (Rio Avion)	
M. dasycneme	MDS	393	M	R: district Moscow	87498
M. emarginatus	MEM	309	M	GR, Th: Kato Stavros	
M. hasseltii	MHA	40	M	MAL: Kuala Selangor	69345
M. horsfieldii	MHO	364	M	MAL: Ulu Gombak	87502
M. montivagus (A)	MMO	257	F	MAL: Ulu Gombak	69340
M. montivagus (B)	MMO	220	M	MAL: Genting Highlands	69344
M. muricola	MMU	37	M	MAL: Ulu Gombak	69339
	MMU	328	M	MAL: Ulu Gombak	87500
M. m. mystacinus	MMS	374	M	E: Navalperal (Rio Tormes)	
M. m. bulgaricus	MBU	319	M	GR, Dr: Paranestion	
	MBU	365	M	GR, K: Loutropigi	
M. nattereri	MNA	318	M	G, B: Pretzfeld	
M. ridleyi	MRI	211	F	MAL: Ulu Gombak	69338

[1] all three specimens were elected as paratypes (see von Helversen et al. 2001).

Data for European species were reported earlier (Volleth 1987). German specimens had been submitted heavily injured or as carcasses by local bat activists to the Department of Zoology at the Erlangen Univeristy in the years 1981 to 1987.

Myotis sp. A of Volleth (1987) is now provisionally assigned to *Myotis mystacinus bulgaricus* Heinrich, 1936. *Myotis* sp. B has meanwhile been described as *Myotis alcathoe* von Helversen et Heller (see von Helversen et al. 2001). Sampling localities for the remaining specimens are given in Table 1.

Results

Description of the basic karyotype of the genus Myotis 2n=44, FNa=52

All *Myotis* species examined by us possess 44 chromosomes. The karyotype is composed of 21 autosomal pairs plus one pair of gonosomes (X,Y). There are three large (1/2, 3/4, 5/6) and one small (16/17) metacentric autosomal pairs. Chromosomal pairs 8 to 15 and 18 to 25 consist of one euchromatic arm only. In contrast to most vespertilionid genera, chromosome 7 possesses a tiny but euchromatic **second** arm. As it is the smaller of the two arms, it is called **short** or **p** arm according to the commonly used cytogenetic nomenclature. The fundamental number of autosomal **euchromatic** arms (FNa) is therefore 52 in *Myotis* compared to FNa=50 in many other vespertilionids. (Chromosomal arms consisting only of heterochromatic material should not be included in the fundamental number.) All chromosomal pairs can be unequivocally distinguished by their G-banding pattern except for the two smallest autosomal pairs, i.e. number 24 and 25, where distinction is possible only in prometaphase spreads. The somewhat larger of both pairs then shows a proximal G-positive band, while the smaller one is completely G-negative. One of these small pairs seems to be bi-armed in most of the species studied. After replication banding (RBG), the short arms present on this pair were shown to replicate late. Only in a few species (see below), these short arms were proven to consist of C-positive heterochromatin. After staining

with fluorescent dyes, however, pairs 24 and 25 are not distinguishable at all. This fact prevents identification of the bi-armed pair by FISH probes in most cases. Exceptions include species with large heterochromatic arms on one of these pairs, e.g. *M. montivagus* (MMO). Fluorescence in--situ hybridization (FISH) with whole chromosome painting probes from a tree shrew (*Tupaia belangeri*, TBE; Volleth et al. 2011) revealed homology of the MMO bi-armed pair to TBE 30 and therefore to human (HSA) chromosome 15. The small pair with HSA 15 homology has been placed on position 24 in the *Myotis* karyogram of Volleth et al. (2002). Since it is in many cases impossible to ascertain the shape of these small pairs with certainty and since the second arm, if present, may consist of heterochromatin, we counted pairs 24 and 25 as single armed for the FNa. In Fig. 4 a comparison of the GTG-, RBG- and CBG-banding pattern for one homolog of each chromosomal pair is shown for *Myotis nattereri*. Karyotype comparison within the family Vespertilionidae showed that despite a large-scale preservation of chromosomal arms, some small differences are present. Chromosomes 1/2, 7, 11, 12, 13, 15, 23 and X exist in two different cha-

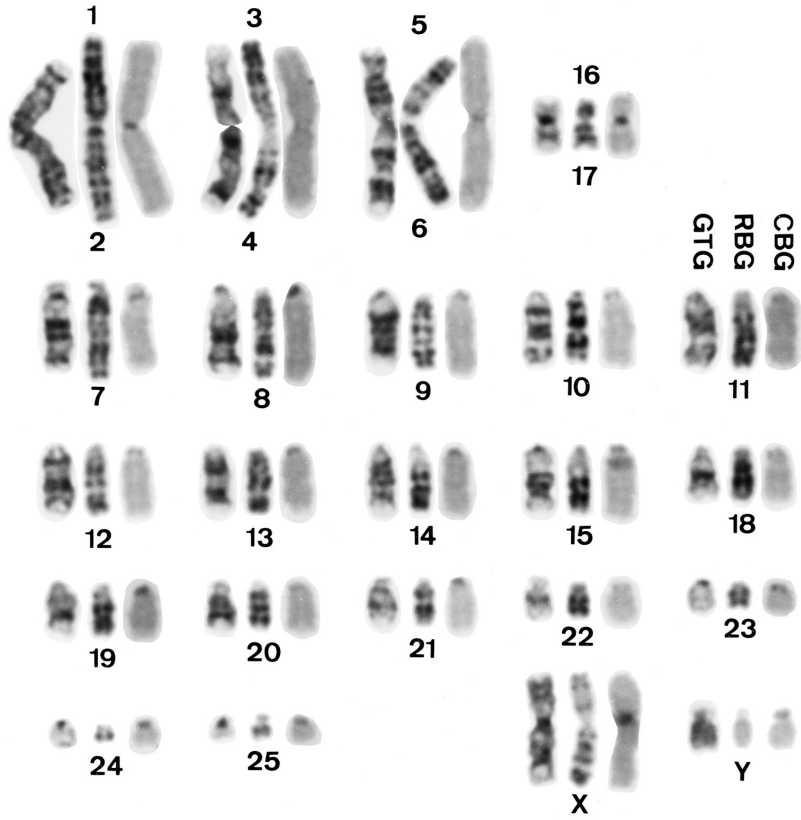

Fig. 4. Comparison of haploid GTG-, RBG-, and CBG-banded chromosomal sets of *Myotis nattereri*. The left chromosome of each triple is G-banded; the chromosome in the middle shows the replication banding pattern after BrdU incorporation and the right chromosome displays the heterochromatic material after C-banding. Please note the euchromatic short arm of chromosome 7 and the heterochromatic segment close to the centromere on chromosome 15.

racter states. One of these character states, state I of chromosome 12, has up to now been found only in the genus *Myotis* and might therefore be considered as an autapomorphy of this genus (Volleth & Heller 1994).

In the species studied here, variations of this basic karyotype are due to the amount and location of heterochromatic material and position and number of the nucleolus organizer regions (NORs).

Species specific features

***Myotis nattereri* (Kuhl, 1817)** MNA

Two males and two females from Germany were studied. All specimens showed a small but distinct intercalary heterochromatic, CBG-positive band on chromosome 15 close to the centromere (Fig. 5). The presence of this heterochromatic segment results in a slightly larger chromosome 15 compared to that of species lacking this band. The large subtelocentric Y chromosome showed a distinct pattern after C-banding (Fig. 6, Table 2).

Four chromosomes have been shown to bear NORs, i.e. number 8, 9, 10 and 14 (Volleth 1987; Table 3).

***Myotis myotis* (Borkhausen, 1797)** MMY

Four males and five females from Germany were studied. As in *M. nattereri*, an intercalary heterochromatic segment was present on chromosome 15 (Fig. 5, Table 2). The long arm of the large, subtelocentric Y chromosome was heterochromatic (Fig. 6). Every acrocentric pair from number 8 to 23 may bear NORs (Volleth 1987).

***Myotis blythii* (Tomes, 1857)** MBL

One male specimen from Greece showed a large, subtelocentric and largely heterochromatic Y chromosome and an intercalary heterochromatic segment on chromosome 15 (Figs. 2 and 3). Nine chromosomal pairs were shown to bear active NORs in this specimen (Table 3).

***Myotis bechsteinii* (Kuhl, 1817)** MBE

Two males were studied, one from Greece and one from Turkey. The medium-sized Y chromosome was submetacentric and largely heterochromatic. The C-banding pattern was only examined in the Greek specimen. This specimen showed a thin but clear heterochromatic band in the proximal part

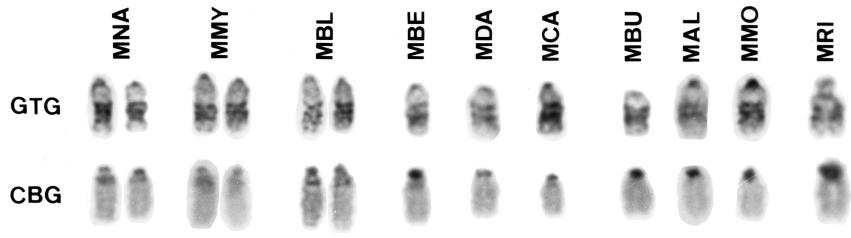

Fig. 5. G-banded (above) and C-banded (below) chromosomes number 15 from ten *Myotis* species. For species abbreviations see Fig. 6. In the case of *M. nattereri*, *M. myotis* and *M. blythii*, both homologs are shown. In these three species the enlargement of the G-negative proximal segment corresponds to the heterochromatic material visible after C-banding.

Table 2. Heterochromatic segments on *Myotis* chromosomal arms and features of the Y chromosome. Species abbreviations as in Table 1 and 4. Y size and morphology: s=small (size of chromosome 25 or smaller); m=medium (size as chromosome 23); l=large (larger than chromosome 23); A=acrocentric; SM=submetacentric; ST=subtelocentric; nk=not known. Only Y chromosomes of MMY and MBL are similar (indicated in bold). i: interstitial location of heterochromatic segment; +: heterochromatic short arm; ++: large heterochromatic short arm

species	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	X	Y
MDS																					s: A
MEM																					s: A
MAL										i											m: SM
MMS										i											s: A
MBU										i								+[1,2]			m: SM
MHO										i											l: A
MHA																		++[1]			l: SM
MMU																		i[1]			m: ST
MMO								i										++[3]			m: ST
MRI		i,+	+	+	+	+	+	+	+			+	+	+	+	+	+	i,++[3]	i, +		nk
MBR										i											s: A
MCA			+																		s: A
MDA																				i	m: SM[4]
MBE		i																+[1]			m: S
MNA									i												l: ST
MBL									i												**l: ST**
MMY									i												**l: ST**

[1] heterochromatic short arm either on 24 or on 25; [2] heteromorphism; [3] assignment proven by FISH; [4] specimen from Spain with large, subtelocentric Y chromosome.

Table 3. Distribution of nucleolus organizer regions (NORs): mean value of NORs per chromosomal pair and cell. Species abbreviations as in Table 1. ID specimen identification number; n number of cells analyzed. The greatest possible value of NORs per chromosomal pair and cell is 2.0. Chromosomal arm number 24 and 25 were counted together as they are not distinguishable by banding pattern, resulting in a maximum value of 4.0

species	ID	n	chromosomal arm number														
			7	8	9	10	11	12	13	14	15	18	19	20	21	22	23	24+25
MDS	393	20				0.9	1.0				1.4	1.8	0.3		0.8		0.8	
MEM	309	21				0.3	1.0		0.9	1.0	1.8	0.7	0.9	0.9		0.9	1.0	1.0
MAL	363	14								1.9					1.9	2.0		
	366	14								1.0			1.1		0.7	1.0	1.0	0.5
	367	24									1.0		1.0		1.9	0.7	1.0	
MMS	374	12	1.0														0.5	4.0
MBU	319	16	1.9	1.7						0.2				0.6		0.8	1.3	
	365	14	1.0	1.0						1.0		0.7				1.3	1.0	
MHO	364	15		1.9						1.9		1.0	2.0		1.0		0.5	
MMU	37	13		1.0								1.0			1.6			
	328	23		2.0								0.9			1.9			
MMO (B)	220	30														1.2		1.8
MMO (A)	257	42														1.0		
MRI	211	11							0.1	0.8		1.7	0.8	1.6		1.0		
MDA	375	18		1.0	1.8	2.0												
MNA	318	12		1.5	2.0	1.9				1.9								
MBL	305	30			1.5	0.8	0.9	0.9		1.3			1.6	0.5	0.4		1.6	

of chromosome 8 and a tiny heterochromatic short arm on both homologs of one of the smallest autosomal pairs (24 and 25). Location of NORs was studied in both specimens. Ten acrocentric pairs from number 8 to number 23 may bear active NORs (Volleth 1987).

***Myotis daubentonii* (Kuhl, 1817)** MDA

Four Greek, three German and one Spainsh specimen were studied. The medium-sized Y chromosome was submetacentric with an euchromatic short and a heterochromatic long arm. An exception was the Spanish specimen with a very large and subtelocentric Y chromosome where the heterochromatic long arm is enlarged compared to the other specimens studied. All specimens showed a large heterochromatic, GTG- and CBC-positive segment in the short arm of the X chromosome adjacent to the centromere, resulting in a clear enlargement of this chromosomal arm (Fig. 6). In addition, on the X chromosome a small heterochromatic segment was found in the proximal part of the long arm. It was GTG-, QFQ- and DAPI-negative and CMA-positive. After CBG-banding, this segment could be proven as CBG-positive only in two specimens. All specimens studied showed NORs only on three chromosomal pairs, i.e. 8, 9 and 10 (Volleth 1987; Table 3).

***Myotis capaccinii* (Bonaparte, 1837)** MCA

One male from Greece was studied. Its Y chromosome was small and acrocentric (Fig. 6). Both homologs of pair 9 showed a heterochromatic short arm (Fig. 7). The staining properties of this additional, late replicating segment are: GTG-, CBG-, QFQ-, and DAPI-positive and CMA-negative. In this specimen, active NORs were found on 12 chromosomal pairs, from number 8 to number 23 (Volleth 1987).

***Myotis emarginatus* (Geoffroy, 1806)** MEM

The single Greek male examined showed a small, acrocentric, heterochromatic Y chromosome (Fig. 6). NORs were found on 11 pairs from chromosome number 10 to 25 (Table 3).

***Myotis dasycneme* (Boie, 1825)** MDS

One male from Russia was examined. The dot-like Y chromosome is thought to be acrocentric (Fig. 6). Seven chromosomal pairs showed active NORs (Table 3).

***Myotis brandtii* (Eversmann, 1845)** MBR

One German male was studied. The Y chromosome was small and acrocentric and only slightly darker stained than euchromatic regions after C-banding. NORs were found on 10 pairs from chromosome number 8 to 23 (Volleth 1987). Chromosomal arm number 16 showed a small heterochromatic band close to the centromere (Table 2).

***Myotis mystacinus mystacinus* (Kuhl, 1817)** MMS

Six German (four males, two females) and one Spanish specimen were studied. The Y chromosome was small and acrocentric (Fig. 6). All specimens examined showed active NORs on four chromosomal pairs, number 7 and 23 to 25 (Volleth 1987; Table 3). A single male showed an extra NOR on one homolog of pair 12.

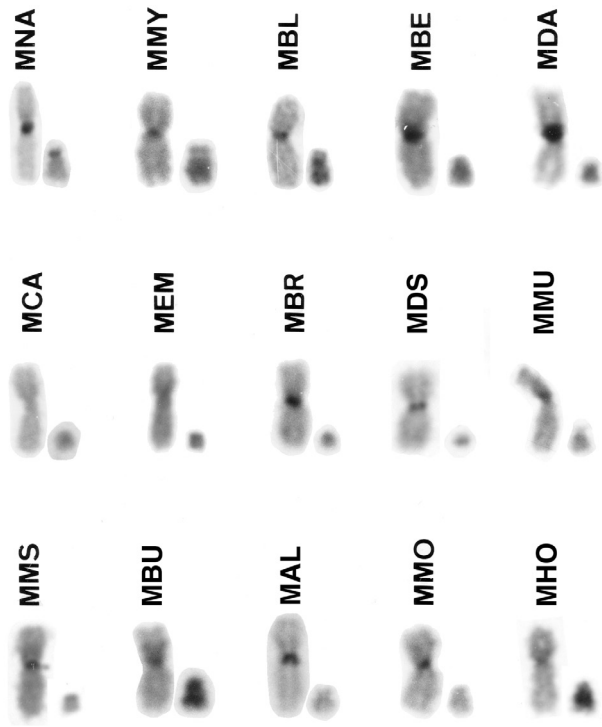

Fig. 6. Comparison of C-banded X and Y chromosomes in 15 *Myotis* species. For further explanation see Results. Species abbreviations: First row: MNA *M. nattereri*; MMY *M. myotis*; MBL *M. blythii*; MBE *M. bechsteinii*; MDA *M. daubentonii*. Second row: MCA *M. capaccinii*; MEM *M. emarginatus*; MBR *M. brandtii*; MDS *M. dasycneme*; MMU *M. muricola*. Third row: MMS *M. m. mystacinus*; MBU *M. m. bulgaricus*; MAL *M. alcathoe*; MMO *M. montivagus*; MHO *M. horsfieldii*.

Chromosomal arm number 16, i.e. the short arm of the small metacentric chromosome, showed a small, heterochromatic segment in the proximal part (Table 2).

***Myotis mystacinus bulgaricus* Heinrich, 1936** MBU

The NORs of this taxon have been described under *Myotis* sp. A in Volleth (1987). As examination of nd1 sequences did not reveal any differences between this taxon and *M. m. mystacinus* (von Helversen et al. 2001), we provisionally assign our specimens to *M. mystacinus bulgaricus* (see Discussion).

Six Greek and one Turkish specimen were studied. The medium-sized Y chromosome was submetacentric and largely heterochromatic (Table 2, Fig. 6). As in *M. m. mystacinus*, chromosomal arm 16 showed a small, proximally situated heterochromatic band. A heteromorphism was found in one of the smallest autosomal pairs. In four of five males, one homolog showed an additional, heterochromatic arm which was of the size of the euchromatic (long) arm. Active NORs were found on chromosomes 7, 8, and 14 to 23 (Volleth 1987; Table 3).

***Myotis alcathoe* von Helversen et Heller, 2001** MAL

The NORs of two specimens were described under *Myotis* sp. B in Volleth (1987). In sum, three females and two males from Greece were studied. The medium-sized Y chromosome was submetacentric and largely heterochromatic (Fig. 6). On arm 16 close to the centromere, a small heterochromatic band was detected. Active NORs were found on three to six different chromosomal pairs per specimen from pair number 14 to 25 (Volleth 1987; Table 3).

***Myotis muricola* (Gray, 1846)** MMU

Two males from Malaysia were studied. The largely heterochromatic Y chromosome was medium-sized and subtelocentric (Fig. 6). One of the two smallest autosomal pairs (24, 25) showed an additional CBG-positive heterochromatic segment close to the centromere. In both specimens, active NORs were found only on three chromosomal pairs, i.e. 8, 18 and 21 (Table 3).

***Myotis montivagus* (Dobson, 1874)** MMO

Two small series of this south-east Asian species were captured in Malaysia at different altitudes, one specimen of each was studied cytogenetically. The sampling locality of the male specimen was located close to the road to the Genting Highlands at about 900 m a.s.l., that of the female in the vicinity of the Field Studies Centre at Ulu Gombak, about 260 m a. s. l. (for details see Heller

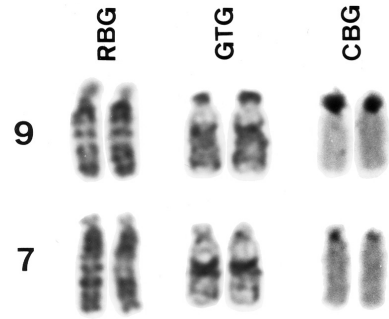

Fig. 7. Comparison of chromosomal pairs number 7 and 9 of *M. cappaccinii*. The heterochromatic short arms of pair 9 are GTG- and CBG-positive in contrast to the euchromatic, GTG- and CBG-negative short arms of chromosome 7.

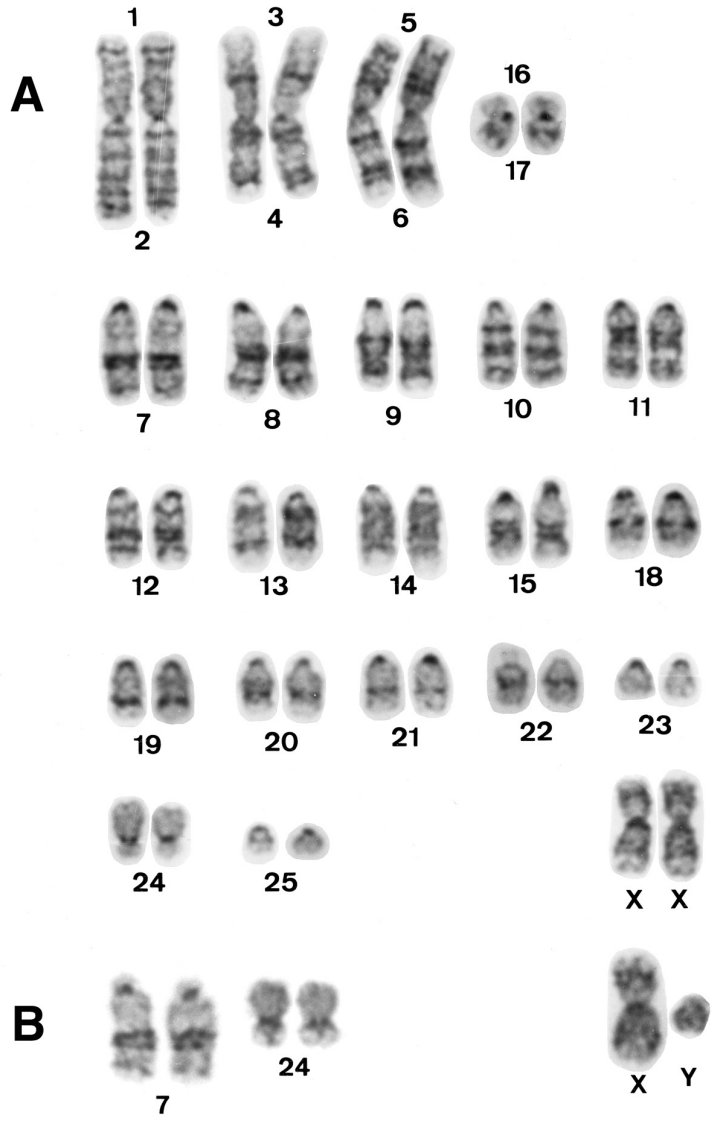

Fig. 8. A: G-banded chromosomal complement of the female specimen A of *M. montivagus*. B: Selected chromosomal pairs of the male specimen B of *M. montivagus*. The acrocentric chromosomal pair number 7 of specimen A represents a derived condition. Specimen B displays the ancestral state of pair 7 for the genus *Myotis*.

& Volleth 1989). Karyotype comparison with other *Myotis* species revealed a distinctly larger chromosome 14 in MMO (see GTG-banded karyogram, Fig. 8). After CBG-banding, a darker stained band was observed in the proximal part of chromosome 14 (Fig. 9). In addition, this region was shown to replicate late. Therefore the increased size of this chromosome is very likely due

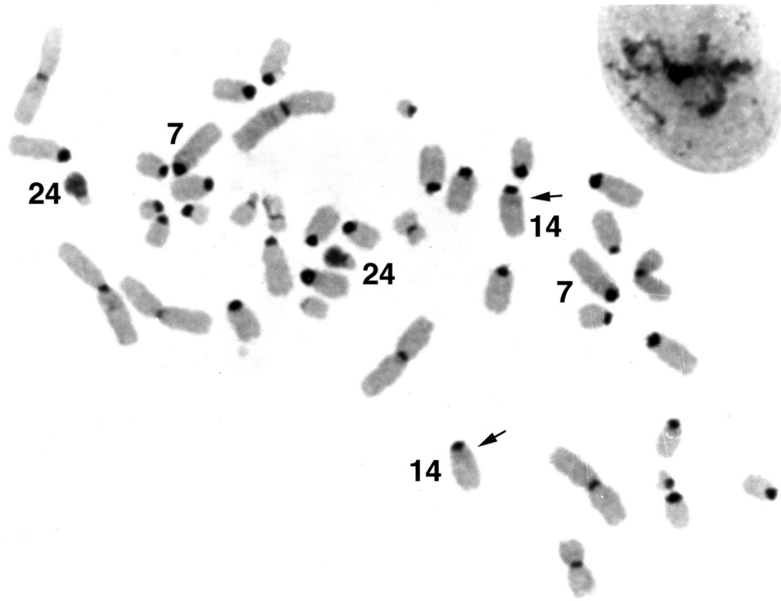

Fig. 9. C-banded metaphase plate of *Myotis montivagus*, specimen A. Please note the heterochromatic second arm on pair 24 and the small heterochromatic band on pair 14.

to addition of heterochromatic material. After CBG-staining, the medium-sized, subtelocentric Y chromosome was only slightly darker stained as the euchromatic parts of the karyotype (Fig. 6). Both homologs of one of the pairs homologous to number 24 or 25 of other *Myotis* species showed an additional large, heterochromatic arm in both specimens studied (Fig. 8). Application of FISH probes revealed that this pair is homologous to number 24.

Two pairs, i.e. number 22 and 25, showed active NORs in the male (Table 3, specimen B). Surprisingly, only a single NOR could be found after AgNOR staining in the female, located on one homolog of number 22 (Table 3, specimen A).

One important feature discriminates the karyotypes of both MMO specimens. In contrast to the male which showed the typical tiny short arm on chromosomal pair 7 (Fig. 8B), the female displayed both homologs of number 7 as structural variants without a short arm but a clearly enlarged proximal region (Fig. 8A). Therefore, an inversion, which led from the plesiomorphic subtelocentric to the derived acrocentric condition in the female, is assumed. Chromosomal rearrangements concerning euchromatic parts of the karyotype are very rarely observed in the genus *Myotis*. This fact points to the possibility that the two specimens studied here belong to two cryptic species. This assumption is supported by the observed 5% sequence divergence in two mitochondrial genes (cyt *b*, nd1) in these specimens (Ruedi & Mayer 2001).

***Myotis ridleyi* (Thomas, 1898)** MRI

Only one female from Malaysia was studied in this species. All chromosomes were bi-armed (Fig. 10). In contrast to all other *Myotis* species described here, chromosomes 8 to 15 and 18 to 23 and 25 showed a small heterochromatic short arm (CBG-positive, GTG- and QFQ-negative).

On the long arm chromosome of 8 a thin but clear heterochromatic band was located in the proximal part. Chromosomal pair 24 is of about the same size as the metacentric pair 16/17 due to a large heterochromatic second arm (assignment confirmed by FISH). Adjacent to the centromere on the long arms of chromosome 24 and 25, a small heterochromatic segment was visible after CBG-staining (Table 2). Active NORs were found on chromosomes 14, 18 to 20 and 22 (Table 3). The NORs were located close to the centromere on the heterochromatic short arms of these chromosomes. In addition, the G-banding pattern of chromosome 11 differed slightly from that of other *Myotis* species.

***Myotis hasseltii* (Temminck, 1840)** MHA

Only a single male from Malaysia was examined. The large, heterochromatic Y chromosome was submetacentric. Both homologs of one of the two smallest autosomal pairs (24, 25) showed a large, heterochromatic short arm (Table 2). Only three metaphase spreads could be examined

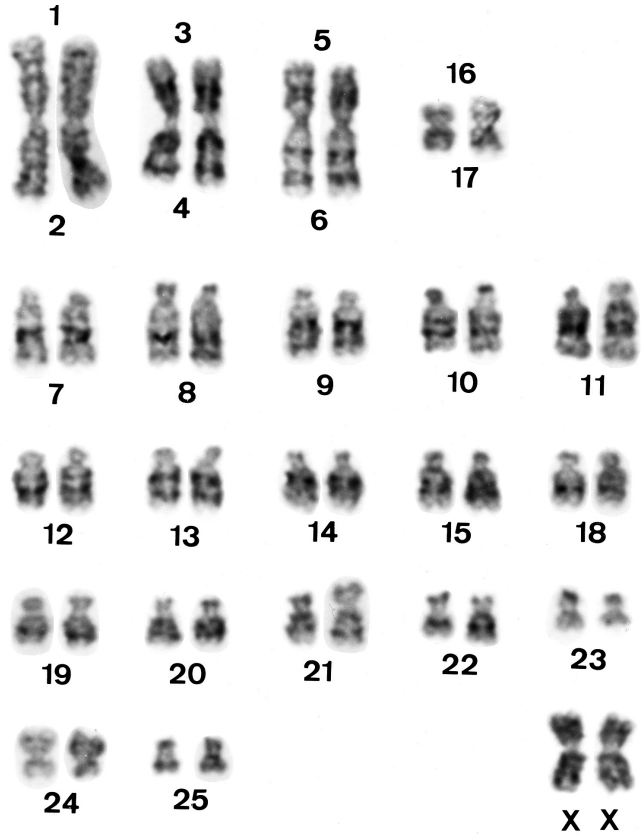

Fig. 10. G-banded karyogram of a female *M. ridleyi*. The short arms of pairs 8 to 15 and 18 to 25 consist of heterochromatic material.

after AgNOR staining and therefore the results should be treated with caution. Four chromosomal pairs showed active NORs, i.e. 8, 11, 14 and 15.

***Myotis horsfieldii* (Temminck, 1840)** MHO

Only a single male specimen from Malaysia was studied. The large, acrocentric Y chromosome consisted to a large extent of heterochromatic material (Fig. 6). Active NORs were found on chromosomal pairs 8, 14, 18, 19, 21 and 23 (Table 3). A small heterochromatic band was found in arm 16 close to the centromere (Table 2).

Discussion

The diploid chromosome number (2n) and the fundamental number of autosomal arms (FNa) have been examined by means of conventionally (i.e. homogeneously or non-differentially) stained preparations in a large number of *Myotis* species. In their comprehensive synopsis of karyotypes of vespertilionid bats, Zima & Horáček (1985) presented chromosomal data and references of all studies published until 1984. Therefore, references dealing with conventionally stained karyotypes of Eurasian *Myotis* are given in Table 5 only if they are originating from 1985 and later.

With only very few exceptions, the *Myotis* karyotype consists of 44 chromosomes. This applies to all 17 taxa presented in this paper. So far, three Asian species have been proven to show a diploid chromosome number larger than 44. A karyotype composed of 46 chromosomes has been reported for *Myotis annectans* from Thailand (Bickham et al. 1986) and *Myotis davidii* from China (Wu et al. 2006; but see recent result of Peng et al. 2011: 2n=44). The increased chromosome number is due to one additional small acrocentric pair which was suspected to be entirely heterochromatic in *M. annectans* (Bickham et al. 1986). Two small additional chromosomal pairs are probably responsible for the increased diploid number of 48 found in the Chinese species *Myotis laniger* (Zhang 1984), which has been included into *M. daubentonii* by Bogdanowicz (1994) but is treated as a separate species in Simmons (2005).

With the exception of the diploid chromosome number, all other karyological characters need the application of banding methods to be accurately determined. In *Myotis*, this is even true for size and shape of the Y chromosome which can safely be determined only by C-banding, displaying the heterochromatic segments. Table 5 lists all karyologically examined Eurasian *Myotis* species and indicates the applied methods.

It turned out that application of two staining methods is advisable for the detection of karyological differences between *Myotis* species. The first technique is the AgNOR or silver-staining, which detects the position of active nucleolus organizer regions (NORs). In the case that the chromosomal pairs can not be distinguished by size and shape, QFQ-banding before or GTG-banding after silver staining has to be applied to the same slide for the assignment of the NOR-bearing chromosomes. Such a double staining procedure is necessary for *Myotis* species. This might be the reason why only few papers have dealt with the description of NORs in the genus *Myotis* (Volleth 1987, Ono & Obara 1994). The results published in these papers are summarized in Table 4, together with our own results presented here for the first time. Most *Myotis* species possess multiple NOR sites located in tiny short arms of acrocentric chromosomes, which are not visible in conventionally or G-banded chromosomes. In *Myotis myotis*, all 14 acrocentric pairs from number 8 to 23 are potential NOR sites. However, only 7.7 active NORs are found on average per cell in any specimen out of the theoretically 28 sites (Volleth 1987). As a consequence, examination of only a single individual will probably not detect all potential NOR sites. For example, the number

Table 4. NOR distribution in the genus *Myotis*. Species abbreviations: MAL *alcathoe*; MBE *bechsteinii*; MBL *blythii*; MBO *bombinus* (published as *nattereri*); MBR *brandtii*; MBU *mystacinus bulgaricus*; MCA *capaccinii;* MDA *daubentonii*; MDS *dasycneme*; MEM *emarginatus*; MFR *frater*; MHA *hasseltii*; MHO *horsfieldii*; MIK *ikonnikovi*; MMA *macrodactylus*; MMS *m. mystacinus*; MMO *montivagus*; MMU *muricola*; MMY *myotis*; MNA *nattereri*; MRI *ridleyi*. N number of specimens examined

chromosome species	7	8	9	10	11	12	13	14	15	18	19	20	21	22	23	24	25	N	reference
MDS				X	X				X	X	X		X		X			1	this paper
MEM				X	X		X	X	X	X	X	X		X	X	X[6]		1	this paper
MAL[1]								X	X		X	X	X	X	X	X[7]		5	Volleth 1987, this paper
MMS	X					X[5]									X	X[8]	X	7	Volleth 1987, this paper
MBU[2]	X	X						X	X	X	X	X	X	X	X			7	Volleth 1987, this paper
MIK[3]	X						X	X						X	X			2	Ono & Obara 1994
MHO		X						X		X	X		X		X			1	this paper
MHA		X			X			X	X									1	this paper
MMU		X								X			X					2	this paper
MMO														X			X[9]	2	this paper
MRI							X	X		X	X	X		X				1	this paper
MMA										X	X	X	X	X	X			5	Ono & Obara 1994
MFR		X	X	X	X		X	X	X	X	X	X	X	X	X			4	Ono & Obara 1994
MBR		X	X	X		X		X		X		X	X	X	X			1	Volleth 1987
MCA		X		X	X	X	X	X	X	X		X	X	X	X			1	Volleth 1987
MDA		X	X	X														8	Volleth 1987, this paper
MBE		X	X	X		X	X	X	X	X			X		X			2	Volleth 1987
MBO	X[4]	X	X	X	X	X	X	X	X		X			X				1	Ono & Obara 1994
MNA		X	X	X				X										3	Volleth 1987, this paper
MBL			X	X	X	X		X			X	X	X		X			1	this paper
MMY		X	X	X	X	X	X	X	X	X	X	X	X	X	X			9	Volleth 1987

[1] *Myotis* sp. B in Volleth (1987); [2] *Myotis* sp. A in Volleth (1987); [3] published as *M. hosonoi* but considered now as a synonym of *M. ikonnikovi* (Kawai in Ohdachi et al. 2009); [4] NOR on chromosome 7 only rarely observed (i.e. low mean value per cell) in the single specimen studied; [5] NOR on only one homolog in a single specimen; 6 NOR on only one homolog of chromosome 24 or 25; [7] NOR on only one homolog of chromosome 24 or 25 in 2 out of 5 specimens; [8] up to 4 chromosomes of pair 24 and 25 with NORs; [9] NORs on pair 25 which is morphologically distinct from pair 24 in MMO.

of 9 chromosomal pairs with active NORs found in the single *M. blythii* specimen examined is unlikely to represent all potential sites for this species.

Examination of rDNA positions in numerous eukaryotes led to the hypothesis that NORs show a high degree of interchromosomal mobility (e.g., in *Allium*: Schubert & Wobus 1985; in Microtidae: Sanchez et al. 1990), probably facilitated by unequal crossing over between adjacent homologous repetitive sequences, although the exact mechanism is still unknown.

As a consequence of the high mobility of rDNA sequences observed also in the genus *Myotis*, the value of distributional patterns of NORs for judging phylogenetic relationships is restricted to few exceptions. The close relationship between *M. m. mystacinus*, *M. m. bulgaricus* and the Japanese representatives of *M. ikonnikovi* (i.e. "*M. hosonoi*"; Ono & Obara, 1994), all showing their own specific distributional pattern for NORs, is confirmed by the exceptional location of NORs within the euchromatic short arm of pair 7 common in these three taxa. On the other hand, the similarity in NOR sites between *M. daubentonii* and *M. nattereri*, which are not closely related according to cytogenetic and molecular genetic studies, seems to be the result of convergent evolution. This assumption is further supported by the different NOR distribution pattern in *M. nattereri* and the closely related Japanese *M. bombinus* (see Table 4, Ono & Obara 1994), formerly included into *M. nattereri*. However, considering a series of specific pecularities in its morphology, Horáček & Hanák (1984) were convinced that *bombinus* represents a separate species. Molecular genetic results (Kawai et al. 2003, Jiang et al. 2010, Tsytsulina et al. 2012) clearly confirmed this view.

Heterochromatic segments consist of densely packed chromatin which resists the chemical processes acting in the C-banding procedure. The unequivocal determination of the chiropteran Y chromosome, which consists to a very small extent of euchromatic sequences but to a species--specific extent of heterochromatin, is enabled only on C-banded metaphase spreads. Table 2 lists Y size and morphology of 16 Eurasian *Myotis* taxa. In five species, the Y chromosome was found to be a very small, probably acrocentric, more or less dot-like chromosome displaying a somewhat darker staining after C-banding than the euchromatic parts of the karyotype. This condition is suspected to be the ancestral one. Out of the species with larger Y chromosomes (see "m" and "l" in Table 2), similar morphology is obvious in *M. myotis* and *M. blythii* only. Three features revealed by C-banding have been found up to now in more than one *Myotis* species (see Table 2). First, a distinct, heterochromatic segment in the proximal part of chromosome 15 has been found in *M. nattereri*, *M. myotis* and *M. blythii*, resulting in an enlarged chromosome compared to other *Myotis* species. The second feature is a small heterochromatic band in chromosomal arm 16, in vicinity of the centromere. It has been found in some whiskered bat species, i.e. *M. m. mystacinus*, *M. m. bulgaricus*, *M. alcathoe* and *M. brandtii*, and in *M. horsfieldii*. The possibility of homoplasic events, however, can not be completely excluded here. The third character is the presence of a short arm on one of the dot-like chromosomes 24 and 25. There might be a very small second arm in several species (Zima 1978, 1979, 1982, Bickham 1979), which, however, is very difficult to detect. In the following, we mention only those cases where a heterochromatic short arm on chromosome 24 or 25 was detected with certainty. A heterochromatic short arm of the same size as the euchromatic arm was found in *M. bechsteinii* and as a heteromorphic feature on only one homolog in some specimens of *M. m. bulgaricus*. Interestingly, such heteromorphism was also reported for *M. ikonnikovi* (Harada & Yoshida 1978, as *M. hosonoi*). From a phylogenetic point of view, the presence of a large heterochromatic second arm in quite a number of Asian species might be important. We found this feature in the karyotypes of *M. montivagus*, *M. hasseltii*, and *M. ridleyi*. The last species, however, shows heterochromatic arms on several chromosomal pairs. In the case of *M. montivagus*, the identity of the chromosome carrying the heterochromatic arm could be proven as pair 24 by FISH (see Results). Similar-sized heterochromatic arms are reported

Table 5. Synopsis of chromosomal data for Eurasian *Myotis* species (alphabetically listed). Additional references for conventionally stained karyotypes published before 1985 can be found in Zima & Horáček (1985). FNa: autosomal fundamental number as given in the referenced studies. Abbreviations: nm not mentioned; nk not known (no males studied); Y shape in brackets: taken from figure (not mentioned in text); p short arm of chromosome; q long arm of chromosome. 24/25: heterochromatic arm either on 24 or on 25. Staining methods: conventional: non-differential staining; G: GTG-banding; C: CBG-banding; NOR: silver staining.

species	2n	FNa	Y shape	heterochromatin addition	NORs	method	reference
alcathoe	44	52	SM			G, C, NOR	this paper
altarium	44	52	A			G, FISH	Ao et al. 2006
	44	50	A			conventional	Gu et al. 2003, Wu et al. 2006
	44	50	A			conventional	Zhang et al. 2010
annectans	46	54	A			G	Bickham et al. 1986
ater	44	50	nk			conventional	Bickham et al. 1986
bechsteinii	44	52	SM			G, C, NOR	this paper
blythii	44	nm	A			G, C	Bickham & Hafner 1978
	44	50	A			conventional	Karataş et al. 2004
	44	50	nk			conventional	Karataş et al. 2008
	44	52	ST	15q	Table 3, 4	G, C, NOR	this paper
bombinus[1]	44	nm	nk			G, C	Harada & Yoshida 1978
	44	50	A		Table 4	G, C, NOR	Ono & Obara 1994
brandtii	44	nm	A			G	Zima 1982
	44	52	A			G, C, NOR	this paper
capaccinii	44	52	A			conventional	Albayrak & Aşan 2002
	44	50	A			conventional	Karataş et al. 2004
	44	52	A	9p	Table 4	G, C, NOR	this paper
chinensis	44	50	A			conventional	Zhang 1984
	44	50	A			conventional	Wu et al. 2006
dasycneme	44	nm	(A)			G, FISH	Kulemzina et al. 2011
	44	52	A		Table 3, 4	G, C, NOR	this paper
daubentonii	44	52	SM	Xp	Table 3, 4	G, C, NOR	this paper
davidii	46	52	A			conventional	Wu & Harada 2006
	44	52	(SM)			G, C	Peng et al. 2011[11]
emarginatus	44	52	A		Tab 3, 4	G, C, NOR	this paper
fimbriatus	44	50	A			conventional	Niu et al. 2007
formosus	44	50	A			conventional	Yoo & Yoon 1992
	44	50	A			conventional	Lin et al. 2002
frater	44	nm	(ST)	number 5p large[2]		G, C	Harada & Yoshida 1978
	44	nm	SM	small A with p		C	Ando et al. 1980
	44	52	ST	24/25p large	Table 4	G, C, NOR	Ono & Obara 1994
gracilis[3]	44	50	A			conventional	Yoo & Yoon 1992

Table 5. continued

species	2n	FNa	Y shape	heterochromatin addition	NORs	method	reference
hasseltii	44	52	SM		Table 4	G, C, NOR	this paper
horsfieldii	44	52	A			G, C, NOR	this paper
	44	50	A		Table 3, 4	conventional	Wu et al 2009
ikonnikovi[4]	44	nm	nk	number 5p heteromorph		G, C	Harada & Yoshida 1978
	44	52	A			G, NOR	Ono & Obara 1994
laniger	48	54	A			conventional	Zhang 1984
latirostris	44	50	A			conventional	Lin et al. 2002
macrodactylus	44	nm	(A)	number 5 p large		G, C	Harada & Yoshida 1978
(Japan)	44	nm	SM	small A with p		C	Ando et al. 1980
	44	52	ST	24/25p	Table 4	G, NOR	Ono & Obara 1994
macrodactylus[5]	44	50	A			conventional	Park & Won 1978
(Korea)	44	52	SM	24/25p small		conventional	Yoo & Yoon 1992
macrotarsus	44	50	A			conventional	Rickart et al. 1999
montivagus B[6]	44	52	ST	14q, 24/25p large	Table 3, 4	G, C, NOR	this paper
montivagus A	44	50	nk	14q, 24p large[7]	Table 3, 4	G, C, NOR	this paper
muricola	44	50	nk			conventional	Bickham et al. 1986
	44	52	ST	24/25q		G, C, NOR	this paper
myotis	44	nm	A			G, C	Bickham & Hafner 1978
	44	nm	nk			G	Zima 1982
	44	50	A			conventional	Karataş et al. 2004
	44	52	ST	15q	Table 4	G, C, NOR	this paper
myst. mystacinus	44	nm	A			G	Zima 1982
	44	52	A		Table 3, 4	G, C, NOR	this paper
myst. bulgaricus[8]	44	52	SM		Tab 3, 4	G, C, NOR	this paper
nattereri	44	52	ST	15q	Tab 3, 4	G, C, NOR	this paper
petax[9]	44	52	A	24/25p small		conventional	Yoo & Yoon 1992
pruinosus[10]	44	52	ST	number 5p large, Xp		G, C	Harada & Uchida 1982
	44	52	A	24/25p, Xp		G, C	Ono & Obara 1994
ridleyi	44	52	nk	numerous p (see Table 2)	Table 3, 4	G, C, NOR	this paper
ricketti	44	52	A			conventional	Wu et al. 2006
	44	50	A			conventional	Zhang 1985
siligorensis	44	52	A			conventional	Harada et al. 1986
taiwanensis	44	50	A			conventional	Lin et al. 2002

[1] *bombinus*: published as *M. nattereri*, but now treated as a separate taxon (Horáček & Hanák 1984, Simmons 2005); [2] chromosome number 5 of Harada is chromosome 24 or 25 according to Bickham's nomenclature; [3] published as *mystacinus gracilis*, being considered now either as a separate taxon or a subspecies of *brandtii* (Simmons 2005); [4] published *as M. hosonoi* but considered to be a synonym of *ikonnikovi* by Kawai in Ohdachi et al. (2009); [5] *macrodactylus* from Korea is listed here separately due to differing chromosomal characters; [6] *montivagus* A, B: see discussion; [7] heterochromatic arm on 24 proven by FISH; [8] *mystacinus bulgaricus* see discussion; [9] *petax*: published as *daubentonii ussuriensis*, but now treated as a separate taxon (Matveev et al. 2005);[10] *pruinosus* additionally shows an inversion on chromosome 1/2; [11] as "*davidii*".

from Japanese specimens of *M. frater*, *M. macrodactylus* and *M. pruinosus* (for references see Table 5). It remains to be shown whether the heterochromatic arm is located on the same dot-like pair (24 and not 25) in all of these species. But even if this was the case, convergent evolution could not be excluded as the observed presence of a tiny short arm in many species might be a prerequisite for further heterochromatin addition.

Based on chromosomal characters, close phylogenetic relationships can be proposed in only two cases. The distributional pattern of NORs, mainly the presence of a NOR on pair 7, points to a close relationship of *M. m. mystacinus*, *M. m. bulgaricus* and *M. ikonnikovi*. From a cytogenetic point of view, however, they are clearly separate species. Due to a heterochromatic segment on chromosome 15, *M. nattereri*, *M. myotis* and *M. blythi* are thought to be closely related. Further, chromosomal analysis confirmed the existence of two cryptic species in the Malayan *M. montivagus* differing by a pericentric inversion on chromosome 7, which took place in the lowland taxon. On the basis of 5% sequence divergence in two mitochondrial genes, the presence of two sibling species was suspected already before by Ruedi & Mayer (2001).

Concluding Remarks

In the present paper we wanted to show that application of various banding techniques is needed to be able to detect karyological differences between *Myotis* species. For unequivocal assignment of heterochromatic bands or nucleolus organizer regions to a certain chromosomal pair, however, sequential staining of the same metaphase plate with two techniques is necessary. In some cases the examination of only a single specimen might not be sufficient to get the broad picture of characters present in a species or population. And, obviously, the number of species studied in such an extensive way is still too small to provide sufficient indications for the phylogenetic relationships in the genus *Myotis*.

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