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Surprising genetic diversity in *Rhinolophus luctus* (Chiroptera: Rhinolophidae) from Peninsular Malaysia: description of a new species based on genetic and morphological characters

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In the family Rhinolophidae, the members of the *trifoliatus* clade are easily recognisable by a unique noseleaf structure and a fluffy fur. Within this group, *Rhinolophus luctus* is the largest species with currently six recognized subspecies, distributed from India to Bali. We investigated genetic (karyotype, mitochondrial DNA sequence) and morphological characters from a Peninsular Malaysian sample. Although the diploid number was 2n = 32 in all specimens, karyotype analysis revealed two largely different chromosomal sets, with a Y-autosome translocation present only in one of the taxa. Morphological examination revealed differences concerning size of the baculum and length of the lower toothrow. Based on these results, a new species is described and the former subspecies distributed on the Malayan Peninsula, *Rhinolophus luctus morio*, is elevated to species rank, *Rhinolophus morio*.

Key words: *R. luctoides*, *R. morio*, *R. trifoliatus*, Y-autosome translocation, synaptonemal complex, baculum, echolocation frequency, FISH, mtDNA

Introduction

In 2005, the number of recognized species of the monotypic family Rhinolophidae (horseshoe bats) was 77 (Simmons, 2005). Since then, at least 19 new species have been described on the basis of morphological differences, corroborated by molecular (12 species) and karyological (one species) data, at present resulting in a total of about 96 species (Yoshiyuki and Lim, 2005; Soisook *et al*., 2008; Wu *et al*., 2008, 2009, 2011; Zhou *et al*., 2009; Benda and Vallo, 2012; Taylor *et al*., 2012; Kerbis Peterhans *et al*., 2013; Patrick *et al*., 2013, Soisook *et al*., 2015). This rapid increase in species number reflects the notion that the general morphological uniformity in the genus *Rhinolophus* masks subtle species-specific differences, which can be recognized only through detailed studies. Representative examples, where species initially have been proposed on the basis of DNA sequence divergences that were subsequently confirmed by morphological data, can be found in South Africa (Taylor *et al*., 2012; Jacobs *et al*., 2013) and South-East Asia (Patrick *et al*., 2013).

Based on morphological features, the genus *Rhinolophus* is divided into 15 groups (Csorba *et al*., 2003). Among these, most easily recognized are the members of the *R. trifoliatus* group by their long, fluffy fur and a unique noseleaf structure with lateral lappets at the base of the sella. This clade, which corresponds to the subgenus *Aquias* Gray 1847 (Guillén-Servent *et al*., 2003), is distributed from the Indian subcontinent to Southeast Asia. The members of the *trifoliatus* group are clearly distinguished by their body size. In addition to the small-sized species *R. sedulus* and the medium-sized *R. trifoliatus*, large-sized members are found throughout the whole distributional range, from Sri Lanka to Nepal on the Indian subcontinent in the

west, to the southern parts of China in the east and north, and to the Indonesian islands Java, Sumatra and Bali in the south. The first large-sized specimen from Java was described as *R. luctus* by Temminck in 1835. However, quite a large number of subspecies or closely related species has been described subsequently, which were all subsumed as subspecies of *R. luctus* by Tate in 1943. A short summary of the complicated history of this taxon can be found in Topál and Csorba (1992). The Indian *R. beddomei*, formerly a subspecies of *R. luctus*, was elevated to species rank for the reason of a different shape of the upper canine and general size differences (Topál and Csorba, 1992). A deviating diploid chromosome number (see below) and smaller body size led Yoshiyuki and Harada (1995) to re-establish the specific rank of *R. formosae* Sanborn, 1939.

However, there are still six different names, which have originally been designated as names for species, subspecies or races but are now all subsumed under the species name *Rhinolophus luctus*. Simmons (2005) accepted *perniger*, *lanosus*, *spurcus* as inhabitants of the northern parts of the distributional range, as well as *luctus*, *morio* and *foetidus* as subspecies of *R. luctus*, whereas *geminus* was considered as synonymous with *luctus*. The assignment of a specimen to a certain *R. luctus* subspecies can presently be done only by the sampling locality as distinct morphological differences have not been described.

The members of the *trifoliatus* clade are not only clearly separated by morphological features from their congeners, but also by a cytogenetic feature, i.e., a low diploid chromosome number (2n). Typically, the genus *Rhinolophus* is karyologically characterized by a high 2n with the majority of species showing a diploid number higher than 56. Apart from the exceptional case of *R. hipposideros* with its three karyotypic variants 2n = 54, 56 and 58 (reviewed in Volleth *et al*., 2013), only a small number of species with a diploid chromosome number smaller than 56 has been reported so far. According to Csorba *et al*. (2003) they belong to four species groups: (1) the *rouxi* group (*R. rouxi* 2n = 56, *R. sinicus* 2n = 36, *R. thomasi* 2n = 36), (2) the *pearsoni* group (*R. pearsoni* 2n = 42 and 44, *R. yunanensis* 2n = 46) and (3) the *euryotis* group (*R. rufus* 2n = 40) (Zhang, 1985; Zima *et al*., 1992; Rickart *et al*., 1999; Gu *et al*., 2003; Ao *et al*., 2007; Mao *et al*., 2007; Wu *et al*., 2009). The fourth group with 2n lower than 56 is the *R. trifoliatus* clade.

Up to now, only conventionally stained chromosomes of two *R. luctus* subspecies have been described. A non-differentially stained karyotype with 2n = 32, a submetacentric X and an acrocentric Y from a single male specimen assigned to *R. l. perniger* was reported by Harada *et al*. (1985) from northern Thailand. From a central Thailand province, a female specimen designated as *R. l. morio* with 2n = 32 was described having a karyotype similar to *R. l. perniger*, however, without presenting a karyotype image (Hood *et al*., 1988). A karyotype comprising 2n = 32 chromosomes has also been reported for *R. beddomei* from India (Naidu and Gururaj, 1984; Koubínová *et al*., 2010). Further, according to the differing diploid number of 52 (Ando *et al*., 1980, 1983), the former *R. luctus* subspecies *formosae* is now treated as a separate species (Yoshiyuki and Harada, 1995). The only species from the *trifoliatus* group for which a differentially stained karyotype has been published is the smallest species of the clade, *R. sedulus*, with a diploid number of 2n = 28 (Volleth *et al*., 2014).

During our chromosomal study of members of the *trifoliatus* group from Peninsular Malaysia, we were intrigued to find two distinctly different chromosomal sets among our '*R. luctus*' sample. Initially, the discovery of an unusual sex chromosome system in the first specimen called for the investigation of additional specimens. The second individual, however, unexpectedly carried a different karyotype. In the present paper we report on morphological, karyological and mitochondrial DNA sequence differences found between these two cryptic rhinolophid species from Peninsular Malaysia. The results show that two forms exist in close geographic proximity, which according to genetic features represent distinct species.

Materials and Methods

Specimens Examined

The specimens were either caught at night with mist nets (SMF 87481, 87485) or at their day roosts (SMF 69288, 69289, 87482–87484) in the years 1984, 1989 and 1992 (SMF indicates accession number of Senckenberg Museum Frankfurt, Germany). Further details, including the collection sites, can be found in Table 1. Preparation of the bacula was performed by maceration of penial tissues for several days in 2–4% potassium hydroxide and clearing them afterwards in glycerol. Cranial measurements were taken to the nearest 0.1 mm using Mitutoyo dial callipers with an accuracy of 0.01 mm.

Specimens are deposited at Senckenberg Museum / Frankfurt. For karyotype comparison, one specimen of *R. trifoliatus* was used. This male (SMF 69284) with a forearm length of 50.9 mm and a body mass of 13.7 g was caught on 23rd March 1984 at Kuala Lompat, Pahang, Peninsular Malaysia.

TABLE 1. Sampling locality and external measurements of specimens examined (in mm). Species assignment according to karyotype except for SMF 69289. SMF — accession number of Senckenberg Museum, Frankfurt; FSC — Field Studies Centre of the University of Malaya, Ulu Gombak; NE — north east. FA — forearm, BM — body mass (g), F3 — 3rd digit, F5 — 5th digit, M3 — metacarpal bone of F3, M5 — metacarpal bone of F5

SMF-No.	GenBank accession	Species	Sex	Locality	Habitat	FA	BM	Baculum length	F3	F5	M3	M5
69288	KR025924	*morio*	♂	Gombak Setia	lowland	65	25.3	3.1	103	86	42	49
87481	KR055700	*morio*	♂	Templer Park, Rawang	lowland	66	–	3.2	104	88	42	50
69289	–	*luctoides*	♂	Genting Highlands	montane	58	24.8	4.1	94	79	41	48
87482	–	*luctoides*	♂	5 km NE FSC	montane	62	21.7	4.8	101	88	42	50
87483	KR055701	*luctoides*	♂	5 km NE FSC	montane	60	23.8	4.3	97	82	39	46
87484	–	hybrid	♂	5 km NE FSC	montane	66	–	4.6	105	91	44	51
87485	KR055702	*luctoides*	♀	Cameron Highlands	montane	64	32.3	–	102	85	38	46

Acoustic Data

Call recordings were made by means of a 6.35 mm microphone (Brüel & Kjaer 4135), measuring amplifier (Brüel & Kjaer 2231) and a high-pass filter (1.3 kHz) on a modified video recorder (frequency response of the system ±3 dB up to 130 kHz). The recordings were re-recorded on a Racal store 4DS tape recorder operating at 152.4 cm/s. The sounds were digitized and analysed using the program Amadeus II (Martin Hairer; www.hairersoft.com).

Chromosome Analysis

From one specimen (SMF 87484) chromosomes were prepared from bone marrow as described in Yong and Dhaliwal (1976). For all other specimens, metaphase spreads were obtained from fibroblast cultures. Cell culture, chromosome preparation, G-banding (GTG: G-bands by Trypsin using Giemsa), C-banding (CBG: C-bands using barium hydroxide treatment and Giemsa), replication banding (RBG: R-bands by bromodeoxyuridine using Giemsa), NOR-staining (Nucleolus Organizer Regions by $AgNO_3$) and fluorescence in-situ hybridization was done as described in Volleth *et al*. (2001, 2009, 2013). In addition to whole chromosome painting probes from *Myotis myotis* (MMY; Ao *et al*., 2006), selected probes from the tree shrew, *Tupaia belangeri* (Müller *et al*., 1999) were used to distinguish segments (a) painted with the probe containing MMY16/17 and MMY21 and (b) being homologous to the proximal (i) and the distal part (ii) of MMY22. Some *Myotis* probes painted two chromosome segments in the *Rhinolophus* species studied here. In these cases, the homology to the proximal (indicated by 'i') and distal ('ii') segments of the respective *Myotis* chromosome was established by comparison of the G-banding pattern with that of MMY and *R. mehelyi*, both previously analyzed using human painting probes (Volleth *et al*., 2002).

Preparation and Staining of Synaptonemal Complex (SC) Spreads

For the preparation of synaptonemal complexes for electron microscopy we followed the methods described in Albini and Jones (1984), Loidl and Jones (1986) and Loidl and Schweizer (1992). Briefly, the tunica was removed from the testes, the testicular tubes were squashed in balanced salt solution and kept on ice. Next, on a clean slide, one drop of the cell suspension obtained was added to four drops of a detergent solution (1% Lipsol in distilled water). The spreading was monitored under phase contrast optics. Afterwards, six drops of fixative (4% paraformaldehyde solution and 3.4% sucrose in water, adjusted to pH 8.2 with borate buffer) were added when the spreading was optimal. The slides were dried over night at room temperature, rinsed in distilled water and stained with silver nitrate. For transformations of slides into electron microscopic preparations see Loidl (1989).

Mitochondrial DNA Analysis

DNA extraction, DNA amplification and sequencing of the partial D-loop was done as described in Wilkinson *et al*. (1997) and Mayer and von Helversen (2001). The sequences were deposited in GenBank (accession numbers are provided in Table 1). *Rhinolophus affinis* (SMF 87480, Templers Park, Rawang,

Malaysia; GenBank U95337) and *R. sedulus* (SMF 89139–89141, Ulu Gombak Field Studies Centre, Malaysia, GenBank U95336; KR025922, KR025923) were used as outgroups. Phylogenetic analysis using the neighbour-joining algorithm was performed as described in Mayer and von Helversen (2001).

Results

The cytogenetic examination of our specimens from Malaysia, which, according to Medway's field guide (1983), would have to be assigned to *R. luctus* ssp. *morio*, revealed the existence of two taxa with distinctly different karyotypes. Concerning the collection sites, one taxon was found in a montane habitat, the other in the lowland, but both locations were in close vicinity to each other and therefore the taxa are considered to be at least parapatric. In order to clarify, which of both taxa should be assigned to *morio*, a comparison with craniodental measurements of the holotypes of *R. l. morio*, *R. l. foetidus* and *R. l. luctus* was undertaken. The results led us to conclude that the lowland specimens belong to the taxon *morio* which is according to cranial and cytogenetic characters elevated to specific rank (see below). Due to differing cranial features, the montane specimens do neither resemble *R. l. foetidus*, distributed in Borneo, nor *R. luctus* ssp. *luctus* whose holotype was collected in Java. Further, also the size of the baculum from a *R. luctus* male (possibly also ssp. *luctus*) from Bali (Heller and Volleth, 1988) differs from our montane specimens. Therefore, a new species is described for our montane sample. Because at first glance the external appearance is not distinguishable from other forms of '*Rhinolophus luctus*', the name '*luctoides*' ('*luctus*-like') was chosen for the new species.

Species description

Rhinolophus luctoides sp. nov.

Holotype

Adult male, SMF 87483, from the vicinity (5 km north-east, approx. 600 m a.s.l.) of the Ulu Gombak Field Studies Centre (3°19'29''N, 101°45'12''E), Selangor, Malaysia, collected on 5th April 1992 by K.-G. Heller and M. Volleth, preserved in alcohol, with skull and baculum extracted, deposited at the Senckenberg Museum, Frankfurt, Germany. The fur was grey-brown with somewhat darker distal parts and silver-grey tips. The penis was 6 mm long and 4 mm broad and covered by long hairs ventrally and dorsally (Fig. 1). The length of the baculum was 4.3 mm. The skull shows the characters of the genus *Rhinolophus*, e.g. distinct rostral inflations and large cochleae (Fig. 2). A comparison of all specimens studied is shown in Fig. 3 for the lower toothrow and in Fig. 4 for the baculum.

Paratypes

The data for two adult males (SMF 69289, SMF 87482) and one adult female (SMF 87485) are given in Tables 1 and 2. Except for SMF 69289, which is preserved as dry skin, they are preserved in alcohol. The skulls of all paratypes and the bacula of SMF 69289 and SMF 87482 have been extracted.

Diagnosis

This species belongs to the *R. trifoliatus* clade, which is recognized by the characteristic lappets at the base of the sella and a woolly appearance of the fur. Concerning the body size, *R. luctoides* is smaller than *R. l. perniger* and *R. l. lanosus*, but of similar size as *R. l. foetidus*, *R. l. luctus* and *R. morio* stat. rev. (see below). It can be distinguished from *R. morio* by the broader penis and the larger baculum. The length of the baculum is larger than 4 mm in *R. luctoides* but smaller than 3 mm in *R. morio*. The lower toothrow (from the canines to the third molar, CM_3) is longer than in *R. morio* and covers a larger part of the mandible. Therefore the ratio of lower toothrow length (CM_3L) to mandible length (ML) is 0.59 or larger in *R. luctoides* and 0.58 or smaller in *R. morio*. With exception of the single female studied, the zygomatic width is smaller in *R. luctoides* than in *R. morio*. The length of the lower toothrow in proportion to mandible length (CM_3L/ML) can also be used to distinguish the taxa *foetidus* and *luctus* from *R. luctoides*, although

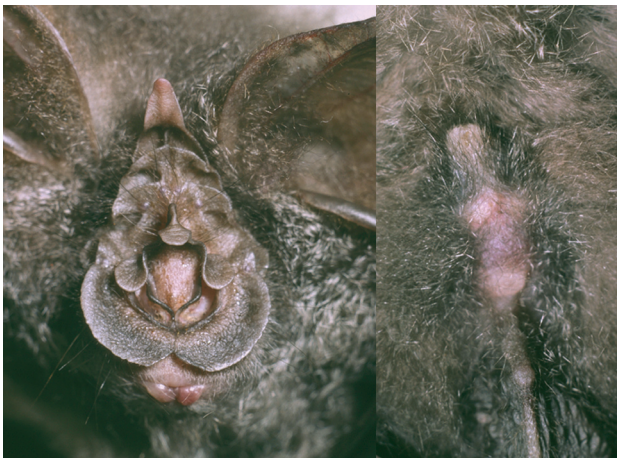

Fig. 1. Facial appearance (left) and penis (right) of the holotype of *R. luctoides* sp. nov.

FIG. 2. Cranium and mandible of *R. luctoides* holotype

they are of similar body size. The ratio CM_3L/ML is shorter in *foetidus* (0.58) but longer in *luctus* (0.63; Table 3).

Although the diploid chromosome number of *R. luctoides* is the same as in *R. morio*, 2n = 32, only six pairs show the same composition of chromosomal arms. The X chromosome of *R. luctoides* is characterized by large heterochromatin blocks which are absent in *R. morio*.

Description

The general appearance of *R. luctoides* is similar to that of *R. luctus* subspecies, and has been described in detail in Csorba *et al*. (2003). In our sample, the forearm length ranged from 59 to 65 mm and the body mass from 21.7 to 32.2 g (Table 1). The baculum size was 4.1 to 4.8 mm and therefore more than 1 mm larger than in *R. morio* (Fig. 4).

Concerning skull length, we found no difference between *R. luctoides* and *R. morio* (Table 2). The same holds true for the length of the mandible with a range of 19.0 to 20.5 mm in *R. luctoides* and 19.2 to 19.5 in *R. morio.* It is therefore surprising that a clear difference was found in the length of the lower toothrow (from the canines to the third molar, CM_3L). This length is smaller than or equal to 11.5 mm in *R. morio* and larger than 11.5 in *R. luctoides*. In Fig. 3 it can be seen that the teeth are covering a larger percentage of the mandible in *R. luctoides* compared to *R. morio*. In the braincase, both species show no differences concerning the mastoid width (MW) but a small difference in the zygomatic width (ZW) with 14.6 mm in *R. morio* and 14.0 to 14.3 mm in male *R. luctoides*. The single female studied showed an extraordinarily large ZW of 15.25 mm.

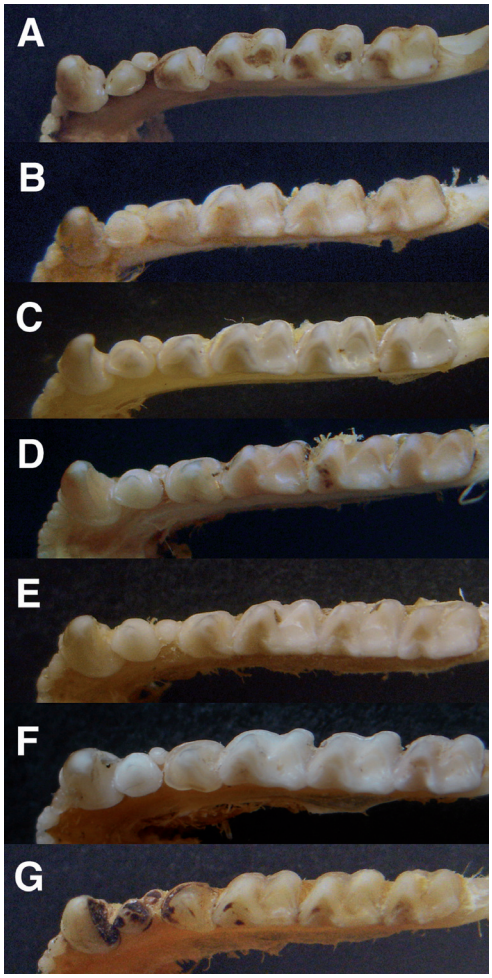

FIG. 3. Comparison of lower toothrows (right mandible) of A, B — *R. morio* and C–G — *R. luctoides*. The position of the middle lower premolar (P_3) – within or outside the toothrow – varies from specimen to specimen. Specimen SMF 69289 (C) is an exceptionally small-sized animal (FA 58 mm). A — SMF 69288, B — SMF 87481, C — SMF 69289, D — SMF 87482, E — SMF 87483, F — SMF 87484, G — SMF 87485

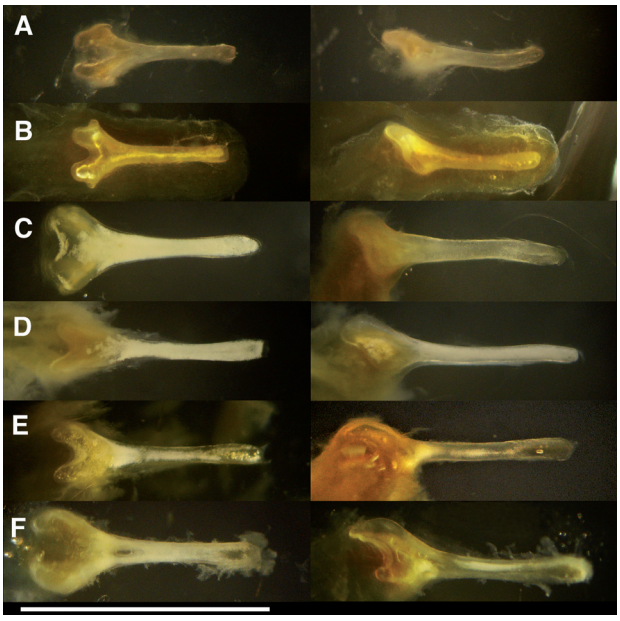

FIG. 4. Dorsal (left) and lateral (right) view of the bacula of *R. morio* (A–B) and *R. luctoides* (C–F). Order of specimens as in Fig. 3. All images are to the same scale (bar = 5 mm)

The upper toothrow length (CM^3L) is, as the lower counterpart, also smaller in *R. morio* (10.55–10.72 mm) than in *R. luctoides* (10.9–11.58 mm).

The constant frequency part of the echolocation call of the hand-held female from Cameron Highlands, Peninsular Malaysia, had a frequency of 42 kHz.

The colour of the fur seems to be unsuitable as a diagnostic character. It ranged from chocolate brown and greyish brown to grey in *R. luctoides* and was brownish and only slightly frosted in our two *R. morio* males. Equally inappropriate as diagnostic character is the position of the lower middle premolar (P_3) because it can be found within or outside of the toothrow in the same species, as has already been stated by Csorba *et al*. (2003: xxix).

Etymology

The name *luctoides* was chosen because this species, regarding external appearance, is very similar to subspecies of *R. luctus*.

Habitat

Rhinolophus luctoides was found in selectively logged Dipterocarp Rain Forest at elevations higher than 600 m, 5 km NE of the Field Studies Centre (FSC) of Ulu Gombak, and in Montane Rain Forest of Genting Highlands and Cameron Highlands. The habitat of the Gombak valley, where the FSC is situated, has been described in detail by Medway (1966). The surroundings of the FSC have been reported as one of the locations with the highest species richness of bats in the Old World (Sing *et al*., 2013).

Rhinolophus morio *Gray, 1842 status revivisco*

The skull dimensions of the holotype of *R. morio* Gray, 1842 from Singapore, deposited in the Natural History Museum London, are similar to those of the two lowland specimens collected by us in the vicinity of Kuala Lumpur (Templer Park, Rawang). Concerning the ratio of lower toothrow to mandible length, the taxon *morio* comes close to subspecies of *R. luctus* (*perniger*, *foetidus*, *lanosus*). However, *morio* differs clearly in the ratio zygomatic width to mandible length from the above mentioned subspecies. In this respect, *morio* resembles other genera in the *trifoliatus* clade, i.e. *R. trifoliatus*, *R. sedulus* and *R. beddomei* (Table 3). By reason of these cranial proportions and the characteristic karyotype with the unique Y-autosomal translocation (see below), we elevate the taxon *morio* to species rank (*Rhinolophus morio* stat. rev.).

TABLE 2. Craniodental measurements. All values refer to mm: SL = skull length, Cond-C = condylo-canine length of skull, ZW = zygomatic width, MW = mastoid width, IW = width of interorbital constriction, C–C = anterior palatal width, M^3–M^3 = palatal breath, C–P^4 = crown length of upper C–P^4, C–P_4 = crown length of lower C–P_4, CM^3L = maxillary toothrow length, CM_3L = mandibular toothrow length, ML = length of mandible

SMF	Species	SL	Cond-C	ZW	MW	IW	C–C	M^3–M^3	C–P^4	CM^3L	CM_3L	C–P_4	ML
69288	*morio*	28.70	25.50	14.65	12.50	3.10	7.73	9.88	4.64	10.70	11.32	4.40	19.5
87481	*morio*	27.90	24.60	14.60	12.35	3.35	7.74	10.3	4.64	10.55	11.15	4.12	19.2
69289	*luctoides*	27.20	23.60	14.05	12.45	3.40	7.64	10.4	4.74	10.85	11.70	4.54	19.1
87482	*luctoides*	28.00	24.50	14.03	12.30	3.74	7.72	10.3	5.00	11.20	12.30	4.90	20.5
87483	*luctoides*	28.35	25.15	14.30	12.60	3.40	8.22	10.5	5.33	11.58	12.25	5.25	19.9
87484	hybrid	29.00	25.40	14.50	13.20	3.50	8.18	10.5	4.78	11.50	12.35	4.80	20.5
87485	*luctoides*	28.25	24.95	15.25	13.00	2.65	8.08	10.8	5.05	11.04	11.60	4.65	19.6

TABLE 3. Size independent cranial ratios CM_3L/ML and ZW/ML in taxa of the *trifoliatus* group. Abbreviations — see Table 2. Measurements in mm. Holotypes are indicated by bold species names. Cranial ratios: CM_3L/ML = relative length of lower toothrow, ZW/ML = relative skull width, for ML, ZW and CM_3L $\bar{x}$ ± SD, minimum and maximum values are given. [1] — values provided by P. D. Jenkins, [2] — values taken from photos provided by P. Kamminga, [3] — this specimen has originally been assigned to *R. l. morio* by Topál and Csorba (2002), *n* — number of specimens, ni — not indicated, NHM — Natural History Museum, London

Species	Locality	Source	*n*	ZW	ML	CM_3L	CM_3L/ML	ZW/ML
morio	Singapore	NHM[1]	**1**	14.93	20.27	11.51	0.568	0.737
morio	Selangor, Malaysia	this study	2	14.63 ± 0.04	19.35 ± 0.21	11.24 ± 0.12	0.581	0.756
				14.60–14.65	19.2–19.5	11.15–11.32		
luctoides	Selangor, Malaysia	this study	**1**	14.30	19.90	12.25	0.616	0.719
luctoides	Selangor, Malaysia	this study	5	14.42 ± 0.50	19.92 ± 0.60	12.04 ± 0.36		
				14.03–15.25	19.1–20.5	11.6–12.35	0.604	0.724
luctoides[3]	Selangor, Malaysia	Topál and Csorba (1992)	1	14.49	20.04	12.11	0.604	0.723
luctus	Java	Naturalis, Leiden[2]	**1**	16.20	20.8	13.04	0.627	0.779
foetidus	Sarawak, Malaysia	NHM[1]	**1**	15.78	21.91	12.79	0.584	0.720
spurcus	Hainan, China	Allen (1928)	**1**	15.5	21.5	12.0	0.558	0.721
lanosus	Kuatun, China	Andersen (1905)	**1**	14.2	22	11.8	0.536	0.645
lanosus	Hunan, China	Zhang *et al*. (2008)	1	14.33	21.44	12.13	0.566	0.668
perniger	India, Nepal	Bates and Harrison (1997)	5–6	15.50 ± 0.6	22.90 ± 0.2	13.00 ± 0.3	0.568	0.677
				14.8–16.2	22.6–23.2	12.7–13.3		
perniger	India, Nepal	Sinha (1973)	6	15.20	21.60	12.60	0.583	0.704
				14.5–15.6	20–22.5	11.8–13.2		
perniger	Myanmar	Bates *et al*. (2004)	3–4	15.45	22.90	13.20	0.576	0.675
				14.4–16.5	22.2–23.6	12.2–14.2		
perniger	India to Thailand	Topál and Csorba (1992)	16–19	15.36 ± 0.77	22.25 ± 0.55	13.0 ± 0.41	0.584	0.690
				13.27–16.45	21.10–23.28	12.35–14.08		
formosae	Taiwan	Yoshiyuki and Harada (1995)	1	11.90	16.60	9.70	0.584	0.717
formosae	Taiwan	Csorba *et al*. (2003)	7	12.75 ± 0.7	17.81 ± 0.51	10.60 ± 0.38	0.595	0.716
				11.83–13.60	16.91–18.33	9.93–11.01		
beddomei	Mysore, India	Andersen (1905)	**1**	14.8	19.7	11.3	0.574	0.751
beddomei	India, Sri Lanka	Soisook *et al*. (2010)	5	13.60 ± 0.7	18.30 ± 0.7	10.50 ± 0.5	0.574	0.743
				12.5–14.5	17.5–19.1	9.9–11.1		
beddomei	India	Bates and Harrison (1997)	3	14.20 ± 0.4	19.10 ± 0.2	10.90 ± 0.2	0.571	0.743
				13.8–14.6	18.9–19.3	10.8–11.1		
beddomei	Sri Lanka	Bates and Harrison (1997)	1	13.10	17.90	10.30	0.575	0.732
beddomei	India, Sri Lanka	Csorba *et al*. (2003)	5	13.66 ± 0.81	18.25 ± 0.53	10.68 ± 0.44	0.585	0.748
				12.35–14.39	17.64–18.86	10.09–11.07		
trifoliatus	Thailand	Soisook *et al*. (2010)	7	11.70 ± 0.4	15.70 ± 0.6	9.10 ± 0.3	0.580	0.745
				11.2–12.5	14.9–16.4	8.6–9.5		
trifoliatus	ni	Csorba *et al*. (2003)	28–36	11.94 ± 0.44	15.93 ± 0.66	9.34 ± 0.39	0.586	0.750
				11.07–12.93	14.87–17.41	8.68–10.29		
trifoliatus	Malaysia, Thailand	Zhou *et al*. (2009)	2	11.2 ± 0.71	15.3 ± 0.71	9.0 ± 0.49	0.588	0.732
				10.7–11.7	14.8–15.8	8.7–9.4		
sedulus	Malaysia, Borneo	Soisook *et al*. (2010)	1–3	9.90 ± 0.4	13.40	7.70 ± 0.5	0.575	0.739
				9.7–10.4		7.1–8.1		
sedulus	Malaysia, Borneo	Csorba *et al*. (2003)	8–10	9.91 ± 0.33	13.08 ± 0.68	7.69 ± 0.49	0.588	0.758
				9.41–10.35	12.24–14.35	7.15–8.74		

Cytogenetic and mtDNA Analyses

Karyotype description of Rhinolophus luctoides *sp. nov. (RLU)*

Three of the four specimens, two males (holotype and paratype) and a female (paratype), were studied cytogenetically. All three specimens showed a karyotype with a diploid number of 2n = 32, an autosomal fundamental number of FNa = 60 and an identical G-banding pattern of all autosomal homologs. There are 15 meta- to submetacentric autosomal pairs ranging from large to small, a large submetacentric X and a large subtelocentric Y chromosome (Fig. 5A). The Nucleolus Organizer Regions (NORs) were detected by silver staining at the secondary constriction close to the centromere of the short arm of chromosomal pair 15. In addition to centromeric heterochromatin, C-banding (CBG) revealed large heterochromatic blocks on X and Y chromosome. The large heterochromatic block on the long arm of the X chromosome is interrupted by a small euchromatic segment (Fig. 6). The Y chromosome shows a minute euchromatic short arm and a very small terminal euchromatic band on the long arm. The other parts of the Y chromosome consist of heterochromatin. Replication (RBG)-banding showed that the large heterochromatic segments on X and Y chromosomes were late replicating.

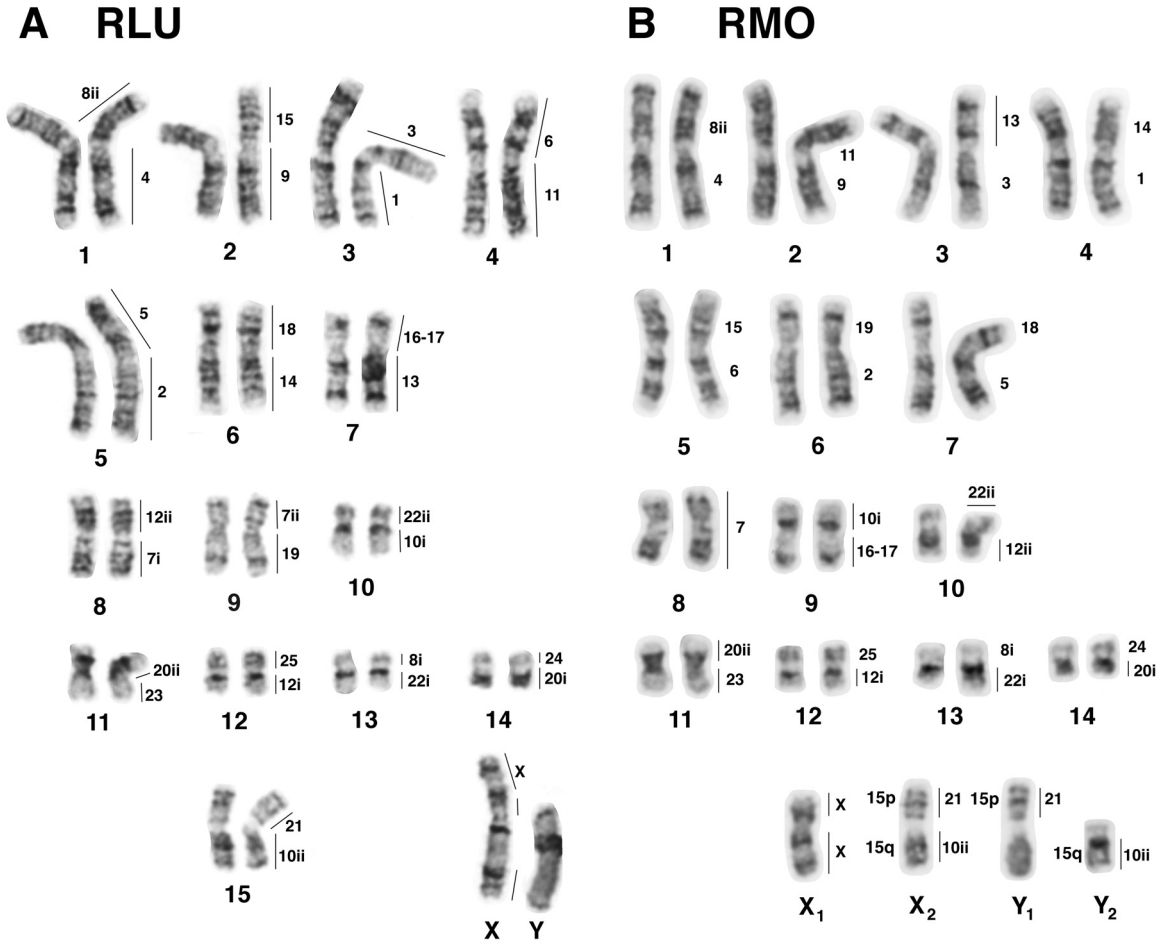

FIG. 5. A — G-banded karyotype of *R. luctoides* sp. nov., male SMF 87483 (holotype). Numbers to the right of each chromosome pair indicate homology to *Myotis myotis* (MMY) chromosomes or chromosomal segments as revealed by FISH with MMY probes and G-band comparison. The appendix 'i' indicates homology to the proximal, 'ii' to the distal part of the respective MMY chromosome (see Materials and Methods); B — G-banded metaphase spread of a male *R. morio*, specimen SMF 87481. Numbers to the right of each chromosome pair indicate homology to MMY revealed by comparison of G-band pattern or, if indicated by a vertical line, by FISH with MMY probes. This species is characterized by a multiple sex chromosome system, $X_1X_1X_2X_2/X_1X_2Y_1Y_2$, resulting from a Y-autosome translocation. Homology to the short (p) and the long (q) arm of chromosome 15, the autosome involved in this translocation, is indicated on the left side of the respective chromosomal arm

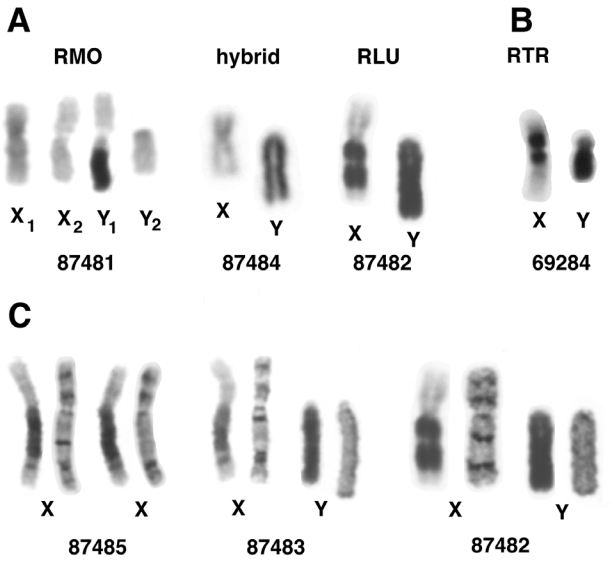

FIG. 6. Comparison of C-banded X and Y chromosomes in the rhinolophid species studied. A — *R. morio* (RMO) left, *R. luctoides* (RLU) right and the specimen presumed to be a hybrid between both taxa in the middle. The Y chromosome of the hybrid was similar to that of RLU whereas the X chromosome resembled that of RMO. The accession numbers of the Senckenberg Museum are given below each set; B — C-banding pattern of X and Y in *R. trifoliatus* (RTR); C — Intraspecific variability in the G-banding pattern in X chromosomes of RLU in spite of similar C-banding pattern (CBG left, GTG right)

Karyotype description of Rhinolophus morio *stat. rev. (RMO)*

Two specimens, collected at about 150 m above sea level (lowland habitat), were assigned to the taxon *morio* according to skull parameters. Both males showed a karyotype composed of 32 chromosomes and a FNa = 60 (Fig. 5B). The karyotype consists of five large metacentric chromosomal pairs, two large submetacentric pairs, four medium-sized meta- to submetacentric pairs, four small metacentric pairs and a single subtelocentric element in addition to the medium-sized submetacentric X chromosome. However, the long arm of one 'homolog' of the NOR-bearing medium-sized pair, being therefore homologous to pair 15 of *R. luctoides*, is nearly completely composed of CBG-positive heterochromatin. Therefore this chromosomal arm was suspected to be of Y chromosomal origin. The G-banding pattern of the long arm of the single subtelocentric element shows homology to the long arm of the autosomal homolog of pair 15. The sex determining system of this taxon can thus be interpreted as a reciprocal Y-autosomal translocation resulting in an even diploid chromosome number in both sexes. Starting from a bi-armed autosome, i.e. chromosome 15, and a subtelocentric Y chromosome with a minute short arm, as found for instance in *R. luctoides*, a Y-autosomal reciprocal translocation would result in two different elements: a Yq/15p translocation product and a Yp/15q bearing element. The second homologue of pair 15 (15p/15q) would remain unaltered. Applying the widely used nomenclature for this $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ sex determining mechanism, X_1 would correspond to the true X chromosome, X_2 to chromosome 15p/15q, Y_1 to the proposed Yq-autosome element Yq/15p and Y_2 to the proposed Yp-autosome element Yp/15q. To support this hypothesis, fluorescence in-situ hybridization (FISH) was performed (see below).

In addition to centromeric regions, CBG-banding detected heterochromatin on chromosome 13, X_1 and Y_1. Only the larger homolog of the heteromorphic autosomal pair 13 showed a C-positive heterochromatic segment in the proximal part of the long arm in both specimens studied. The X chromosome displayed only a slightly enlarged pericentric heterochromatic region, whereas nearly the complete Yq-bearing arm of the Y-autosome translocation product (Y_1, Yq/15p) consisted of heterochromatin (Fig. 6).

Silver-staining confirmed that the secondary constriction close to the centromere in 15p is indeed a Nucleolus Organizer Region (NOR). In one specimen, both NORs were active, in the other specimen only that located at X_2 (15p/15q) showed silver grains.

Karyotype Description of a Suspected RLUxRMO Interspecies Hybrid

A comparison of the RLU (*R. luctoides*) and RMO (*R. morio*) karyotypes on the basis of G-banding and FISH results (see below) revealed that only six pairs showed the same composition of chromosomal arms (pair 1 and pairs 11 to 15). The remaining nine autosomal pairs (2 to 10) differed in arm composition between the two taxa. Pairs 2 and 5 of *R. morio* could be transformed into pairs 2 and 4 of *R. luctoides* by a whole arm reciprocal translocation (WART). For the remaining pairs, however, even more complex rearrangements, for example serial WARTs, would be necessary to transform the *R. morio* karyotype into that of *R. luctoides*.

These results enabled the analysis of the enigmatic karyotype of another male specimen, collected in the montane habitat of *R. luctoides*, from

which only metaphase spreads obtained from bone marrow could be studied. Out of the 30 G-banded autosomal elements only 12 could be arranged into pairs (Fig. 7). From the remaining 18 autosomal elements, nine show the G-banding pattern of *R. luctoides* and nine that of *R. morio*. The X chromosome also resembles very much that of the lowland taxon, RMO, whereas the Y chromosome is large and almost completely heterochromatic as in RLU males (Fig. 6). Therefore this specimen is very likely a F1 hybrid between a *R. morio* female and a *R. luctoides* male. During gametogenesis, six bivalents, one quadrivalent and a ring consisting of 14 different elements would be expected to form in this hybrid. Such a situation is prone to result in frequent production of unbalanced gametes or meiotic arrest, leading to reduced fertility or even sterility.

Karyotype Description of Rhinolophus trifoliatus *(RTR) Temminck, 1834*

For comparison, the karyotype of the closely related trifoil horseshoe bat is also shown. *R. trifoliatus* is clearly distinguished from the above mentioned taxa by its smaller body size and the characteristic yellow colour of the noseleaf.

The diploid chromosome number of the single male studied of *R. trifoliatus* was also 2n = 32 with FNa = 60 (Fig. 8). All chromosomes are bi-armed and with few exceptions (see below) the G-banded autosomal complement is similar to that of *R. morio*.

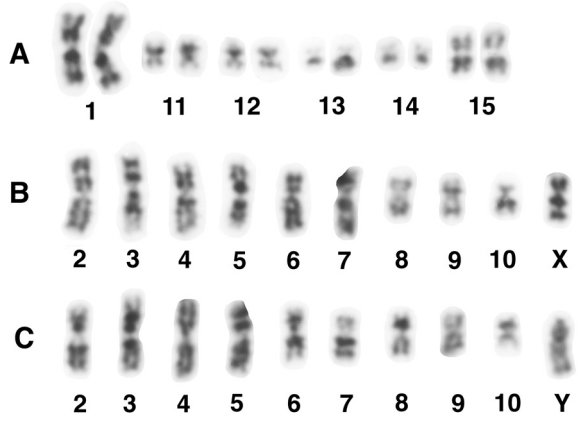

FIG. 7. Chromosomal complement of specimen SMF 87484, assumed F1 hybrid of a RLU male and a RMO female. A — Chromosomal pairs with similar banding pattern in both taxa are aligned as pairs in the hybrid; B — Chromosomes with similar banding pattern to number 2 to 10 and X of RMO were found as single elements in the hybrid; C — Chromosomes with similar banding pattern to number 2 to 10 and Y of RLU were also found only once. In sum, this is a balanced karyotype consisting of 32 chromosomes

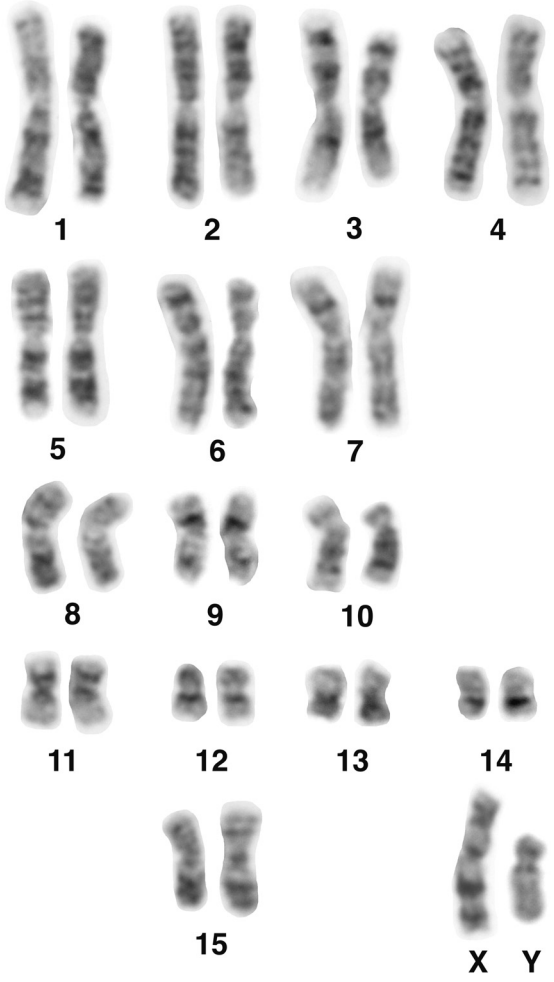

FIG. 8. G-banded karyotype of *R. trifoliatus*, male SMF 69284. The pairs have been arranged in the same order as in RMO according to their G-banding pattern

The X chromosome is a metacentric element with a large pericentromeric C-band positive heterochromatic segment and therefore similar to that of the closely related *R. sedulus* (2n = 28 — Volleth *et al*., 2014). The large submetacentric Y chromosome consists of C-band positive heterochromatin except for the euchromatic distal half of the short arm (Fig. 6). Chromosome RTR10 shows a small interstitial heterochromatic band in the long arm of both homologues. A submetacentric NOR-bearing chromosome corresponds to the metacentric chromosome 15 of *R. luctoides*. The difference can be explained by a small pericentric inversion, which could be confirmed by FISH.

Fluorescence In-situ Hybridization (FISH)

The correspondence of chromosomal arms of *R. luctoides* (RLU) to the chromosomes of the

vespertilionid bat *Myotis myotis* (MMY) was traced by hybridizing all MMY whole chromosome painting probes onto metaphase preparations from *R. luctoides* specimens. Correspondence to MMY chromosomal segments is indicated in the *R. luctoides* karyogram (Fig. 5A). In most cases, a single MMY chromosomal arm corresponded to a single RLU arm. As in other rhinolophids, homology to certain *Myotis* chromosomes, i.e., MMY 7, 8, 10, 12, 20 and 22, was found on two *R. luctoides* chromosomal arms each.

In *R. morio* (RMO), only the most informative six MMY probes could be used for FISH experiments due to limited material available (Fig. 5B). These experiments showed that compared to *R. luctoides* the composition of chromosomal arms is different in RMO8, RMO9 and RMO10, but identical in RMO11 to 14. Chromosome 15 in *R. luctoides* was shown to be homologous to MMY21 in the short arm and to MMY10 in the long arm (Fig. 9A). For this reason, both probes were also used to confirm the Y-autosomal translocation between chromosome 15 and the Y in *R. morio*. As expected, MMY 21 painted the short arms of RMO X_2 and Y_1. Furthermore, the long arm of RMO X_2 (i.e. the 15p/15q homolog) and the long arm of Y_2 (i.e. the subtelocentric Yp/15q) showed homology to MMY10 (Fig. 5B and 9A). The unpainted segments, the long arm of RMO Y_1 and the short arm of Y_2 are thus presumed to carry Y specific sequences.

To demonstrate the pericentric inversion in chromosome 15 of *R. trifoliatus*, FISH with a probe containing MMY21 was performed. Indeed, hybridization signals were not only found in the short arm, but also in the proximal part of the long arm, distally to the NOR (Fig. 9B).

Synaptonemal Complexes

Synaptonemal complexes (SCs) are proteinaceous structures, which mediate the pairing of homologous chromosomes during prophase of the first meiotic division. The number of SCs corresponds to the haploid number of chromosomes. However, the length of the SC built by the sex chromosomes, X and Y, varies during pachytene and comprises only homologous sequences found at the small pseudoautosomal regions. The remaining parts, where no SCs are formed, appear thickened (Zickler and Kleckner, 1999). SCs in one male of *R. morio* (SMF 87481) were analyzed to study the behaviour of the chromosomes involved in the Y-autosomal translocation in meiotic prophase and to identify the regions of synapsis between X_1 and Y_1 or Y_2. The silver-stained microspreads of pachytene spermatocytes of this male showed 14 autosomal bivalents and one multivalent. According to the chromosomal analysis, four elements are expected to form this multivalent: the X chromosome (X_1), the autosome corresponding to pair RLU15 (X_2), and both translocation elements between the autosome and the Y chromosome, Y_1 and Y_2. Synaptonemal complexes were formed by the autosomal parts of these elements, while the asynapsed gonosomal parts, i.e. X_1 and the long arm of Y_1, appeared thickened.

In the centre of the quadrivalent no SC was visible because of the presence of an NOR on the short arms of X_2 and Y_1, close to the centromere. One terminus of X_1 was found in close vicinity to the kinetochores of the other three elements. This can be interpreted as synapsis either with Y_1 or Y_2. Figs. 10A, B and C display this 'X'-like configuration of the quadrivalent. Only in few spreads a terminal end-to-end association of X_1 and Y_1 was found (Fig. 10D).

Mitochondrial Sequences

Partial DNA sequences of the highly variable D-loop were analysed from two *R. morio* and two *R. luctoides* specimens. These sequences were compared with our own data from three *R. sedulus* and one *R. affinis* specimen from Malaysia. The genetic distance of *R. morio* to *R. luctoides* was on average 7.8%. In contrast, the genetic distance within *R. morio* and *R. luctoides* was only 0.5% and 3.6%,

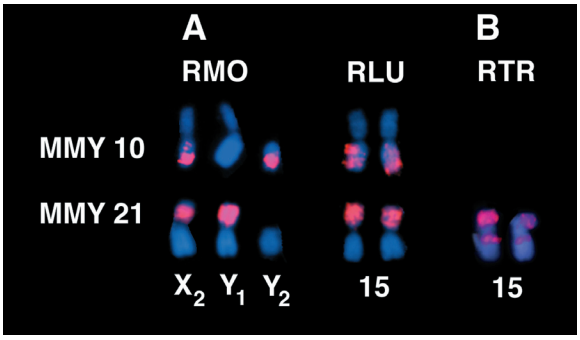

FIG. 9. Results of FISH experiments with MMY probes homologous to pair 15. A — In RLU (right), MMY10 painted the long arm and MMY21 the short arm of both homologs of pair 15. A similar pattern was found only on one chromosome (X_2) of RMO (left). As a result of the Y-autosomal translocation, homologous sequences to MMY21 are localized on the short arm of Y_1, and the long arm of Y_2 harbours sequences with homology to MMY10; B — Confirmation of the pericentric inversion in RTR shown by the hybridization pattern of MMY21

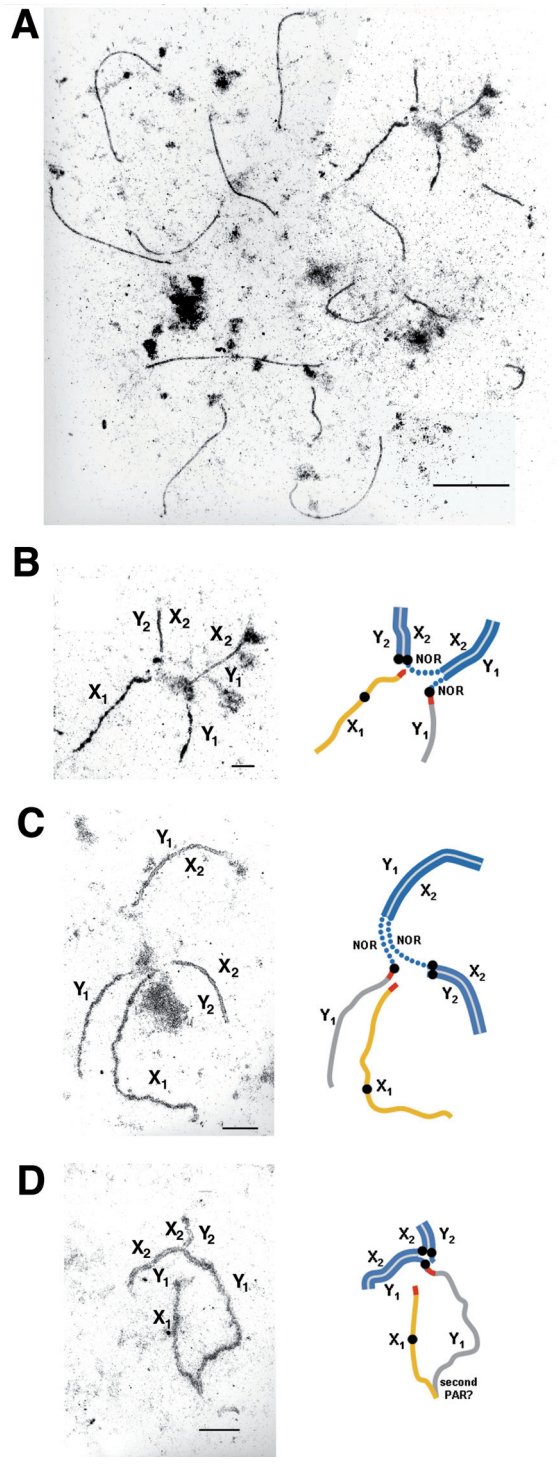

FIG. 10. A — Full set of synaptonemal complexes (SC) from a microspread pachytene spermatocyte of *R. morio* male SMF 87481; B — Enlargement of the quadrivalent displayed in A (left) and schematic presentation (right). The autosomal parts of X_2, Y_1 and Y_2 are fully synapsed, while X_1 and the gonosomal part of Y_1 appear asynapsed and thickened. The presence of an NOR close to the centromere in X_2 and Y_1 led to interruption of the SC in the centre of the quadrivalent; C — Quadrivalent of another cell with a similar configuration; D — In this quadrivalent, a small synapsed region is present in the distal part of X_1 and Y_1. Putative pseudoautosomal regions are indicated in red. (Bar in A — 10 µm, in B–D — 2.5 µm)

respectively. The genetic distance of *R. sedulus*, a species also belonging to the *trifoliatus* clade, to *R. morio* and *R. luctoides* was 12% and 10%, respectively. All three species showed about 20% difference to *R. affinis*.

Mitochondrial ND1 sequences from the hybrid specimen SMF 87484 showed a genetic divergence of only 0.5% to *R. morio*, but of 5.8% to *R. luctoides* (unfortunately the sequences were lost, only the results of the analysis were kept). These results imply that the hybrid specimen carried mitochondrial sequences of *R. morio* and therefore had a *R. morio* mother. This is in full agreement with the hypothesis based on the chromosomal study.

DISCUSSION

We investigated the karyotypes of six Malaysian specimens initially classified as *R. luctus*, using the classical banding techniques (GTG, CBG, RBG and NOR-staining; see Materials and Methods for abbreviations). In addition, we performed Zoo-FISH using the entire set of whole chromosome painting probes from the vespertilionid species *Myotis myotis*. According to the collection site and the currently established taxonomic classification, our specimens should be assigned to the subspecies *R. l. morio* (Chasen, 1940; Medway, 1983), as the type locality of this subspecies is Singapore (Gray, 1842). However, despite an identical diploid chromosome number and FNa in the specimens studied, we found two distinctly different karyotypes with nine bi-armed pairs differing in arm composition. The pronounced karyotypic differences clearly indicate that the sample analysed here comprise two cryptic species. Notably, the smallest geographic distance between the collection sites of the two taxa was less than 15 km and therefore they can be described at least as being parapatric. On the other hand, the difference in altitude along the valley of the Gombak River of about 500 m between the collection sites may represent an important separating factor. Other examples for elevational preferences in bats are the two morphotypes (or possibly sibling species) of *R. arcuatus* found in the Philippines (Ingle and Heaney, 1992; Sedlock and Weyandt, 2009), and also *Murina* species from Taiwan (Kuo *et al*., 2014).

According to our morphological comparison with the respective holotypes, we conclude that only our lowland specimens represent *R. morio*, whereas the montane specimens, found at an altitude of about 600 m on the slopes of the Gombak valley and at

about 1,400 m on Cameron Highlands, are representatives of a yet unidentified taxon. For this latter taxon, we consider the name *R. luctoides* appropriate for the reason of similarity in external appearance with *R. luctus*.

The karyotype of our specimens assigned to *R. morio* (RMO) is very similar to the karyotype of the single so far studied specimen of *R. trifoliatus* (RTR). The karyotype of *R. trifoliatus* differs from *R. morio* only in the presence of a large heterochromatic segment on the X chromosome and an inversion on the NOR-bearing autosomal pair, which could be clearly demonstrated by FISH. The homologous pair is involved in a Y-autosome translocation in *R. morio*. In contrast, the karyotype comparison of *R. morio* and *R. luctoides* (RLU) revealed not only that the Y-autosome translocation is absent in the latter but also that nine bi-armed autosomes differ in the composition of chromosomal arms. A whole arm reciprocal translocation (WART) between chromosomal pairs RLU2 and RLU4 would result in pairs RMO2 and RMO5. The remaining seven chromosomal pairs, however, show monobrachial homology but cannot be aligned by simple WARTs. During gametogenesis in a hybrid specimen the autosomes would have to form a quadrivalent and a ring of 14 elements, in addition to six bivalents. Similar configurations in other mammalian species usually result in highly infertile or even sterile hybrid animals (reviewed in King, 1993). One could therefore speculate that both taxa would probably not have survived as genetically distinct entities in such close geographic proximity (para- or sympatry) without these profound cytogenetic differences. In line with our findings, it has recently been shown that sympatric sister species in rodents are chromosomally more differentiated than allopatric ones. The same karyotype was found in 50% of allopatric species but only in 10% of sympatric taxa (Castiglia, 2014).

Sex Chromosome System

Gonosome-autosome rearrangements are rare events in mammalian karyotype evolution because they cause serious perturbations of gametogenesis (Veyrunes *et al*., 2007).

In the case of a fusion between an autosome and the X chromosome, the resulting sex chromosome system is described as XX/XY_1Y_2 with a diploid number of 2n in females and 2n+1 in males. Y_2 represents the non-fused autosome present only in males. In Chiroptera, this type of rearrangement was found in the vespertlionid *Glischropus tylopus* (Volleth and Yong, 1987; Volleth *et al*., 2001), and also in Phyllostomidae, where it is present in a large number of species of Stenodermatinae (e.g., Pieczarka *et al*., 2013) and in the genus *Carollia* (e.g., Tucker and Bickham, 1986; Pieczarka *et al*., 2005). If not the X but the Y chromosome is fused with an autosome, the diploid chromosome number in females (2n) is higher than that of males (2n-1). The description used for such a system is $X_1X_1X_2X_2/X_1X_2Y$ with X_2 being the autosome and Y the fusion product between the original Y and the autosome. A sole Y-autosome fusion has been described for example in species of spiral-horned antelopes (*Tragelaphus* — Rubes *et al*., 2008), but not yet proven in Chiroptera. However, Y-autosome fusions in combination with X-autosome fusions, a so-called NeoXY system has been found in several genera of the Stenodermatinae (e.g., Tucker, 1986; Noronha *et al*., 2010; Pieczarka *et al*., 2013).

Even more rarely found is a translocation between an autosome and the Y chromosome. As the diploid chromosome number is not altered by this rearrangement, banding studies or even FISH analyses are necessary to detect such a multiple sex chromosome system, which is described as $X_1X_1X_2X_2/X_1X_2Y_1Y_2$. One of the rare examples is the silvered leaf monkey, *Trachypithecus cristatus*, where FISH with human probes delineated a reciprocal translocation between the largest autosome, TCR1, and the Y chromosome (Bigoni *et al*., 1997; Xiaobo *et al*., 2013). The underpinning mechanism in this case, however, is not a simple translocation, but a complex rearrangement which has been detected using partial human paints (Xiaobo *et al*., 2013). Unfortunately, the interesting question which meiotic configuration will be formed in this species remained unanswered, as no meiotic studies have been undertaken.

A second example of the rare $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ sex chromosome system has been described in the New World monkey genus *Alouatta* (Atelidae). In this species-rich genus, Y-autosome rearrangements have been reported in all species studied so far (Steinberg *et al*., 2014 and references therein). However, different kinds of rearrangements have been observed, ranging from a 'simple' Y-autosome fusion to a complex rearrangement involving two different autosomes. For example, in the $X_1X_1X_2X_2/X_1X_2Y_1Y_2$ sex chromosome system found in *Alouatta carraya*, the male karyotype comprises four non-homologous elements which should form a quadrivalent in meiosis to enable balanced

transmission into the resulting gametes. Pachytene studies indeed confirmed the presence of a quadrivalent with synaptonemal complex formed by the respective homologous autosomal regions and a short region of synapsis between X_1 and Y_1 at the pseudoautosomal region (PAR) (Rahn *et al*., 1996; Mudry *et al*., 2001). Interestingly, it has been shown for *Alouatta* that a multiple sex chromosome system evolved independently in two geographically separated species groups (Steinberg *et al*., 2014), as in each group a different autosome was involved in the Y-autosome rearrangement.

This is a remarkable parallel to the situation found in *R. morio* studied here and the closely related *R. sedulus* (Volleth *et al*., 2014). Both species display an $X_1X_1X_2X_2$/ $X_1X_2Y_1Y_2$ sex chromosome system but clearly differ in the autosome involved. The synaptonemal complex (SC) analysis of pachytene nuclei from a *R. morio* male confirmed the presence of a multiple sex chromosome system. In addition to 14 bivalents formed by the autosomal pairs, a multivalent was found. The interpretation of this fragile structure, however, is complicated by the presence of Nucleolus Organizing Regions (NORs) on the autosome (X_2) and one translocation element (Y_1). The particular structure of the NOR with its less condensed condition resulted in the fact that a SC was not visible at the NORs in the pachytene nuclei. One terminus of the X_1 chromosome was found in the vicinity of the kinetochores of the autosomal SCs in all pachytenes examined. We therefore suspect the presence of a PAR either on Y_1 or on the short arm of Y_2. Rarely, the other end of X_1 was found in contact with the terminal end of the heterochromatic arm of Y_1, which is possibly homologous to the long arm of the original Y chromosome. This observation points to the possible existence of a second PAR, as is found in human X-Y pairs.

DNA Analyses

Molecular studies revealed that *Rhinolophus* species may roughly be divided into two large groups, i.e. an African-European clade and an Asian clade (Guillén-Servent *et al*., 2003; Stoffberg *et al*., 2010; Foley *et al*., 2015). Molecular phylogenies reconstructed from comparative mitochondrial (cytochrome *b*, COI, D-loop) or nuclear (RAG1, introns of TG, PRKC1 and THY) sequence analyses point to a basal position of the *trifoliatus* clade in the rhinolophid tree (Guillén-Servent *et al*., 2003; Francis *et al*., 2010; Agnarsson *et al*., 2011; Patrick *et al*., 2013) or to a position at the base of the Australasian branch (Stoffberg *et al*., 2010, only *R. formosae* studied; Foley *et al*., 2015). Concerning the relationships within the *trifoliatus* clade, the closest relative to any specimen of '*R. luctus*' was *R. trifoliatus* in the majority of studies (Guillén-Servent *et al*., 2003; Francis *et al*., 2010; Sazali *et al*., 2011). Only Agnarsson *et al*. (2011) reported *R. sedulus* as the sister species of *R. trifoliatus*, a view which is corroborated by the similarity of X chromosomal morphology in *R. sedulus* and *R. trifoliatus*. Based on cytochrome *b* sequences, Sazali *et al*. (2011) reported a genetic divergence between *R. trifoliatus* and '*R. luctus*' from Malaysia of only 3.7%. In the same study, the Malaysian '*R. luctus*' were found to diverge from *R. sedulus* by 7.1%, and from all other *Rhinolophus* species by more than 10%.

The results of the mt D-loop analyses presented here clearly identified two genetically distinct groups within our sample showing an average sequence difference of 7.8%. These results are in full agreement with our cytogenetic data, where the lowland and montane taxa, *R. morio* and *R. luctoides*, respectively, could also be well-defined karyologically. Both *R. morio* specimens, living in close vicinity, showed only a genetic distance of 0.5% in comparison to 3.6% difference between *R. luctoides* from Cameron Highlands and Ulu Gombak. A comparison with D-loop sequences deposited in GenBank surprisingly revealed that the smallest difference to *R. luctoides* is found in '*R. luctus*' specimens from China (Hubei and Sichuan provinces — Li *et al*., 2006; Xu *et al*., 2012). According to the phylogeny presented in Fig. 11, based on partial D-loop sequences, the two Malaysian species, *R. morio* and *R. luctoides*, are not each others' closest relative but are separated by '*R. luctus*' specimens from China and Myanmar. Unfortunately, G-banded karyograms are not available so far for specimens from these geographical regions. Future studies will be required to confirm the two hypotheses we propose here, based on our genetic data: 1) *R. morio*'s closest relative might be *R. beddomei* from India whereas that of *R. luctoides* is distributed in China, and 2) *R. morio* and *R. luctoides* might possibly occur in sympatry also in other regions of South East Asia.

Taxonomy

Body size, reflected by forearm length, and absolute cranial dimensions show a broad range among the taxa, which originally were subsumed under '*Rhinolophus luctus*'. Museum collections

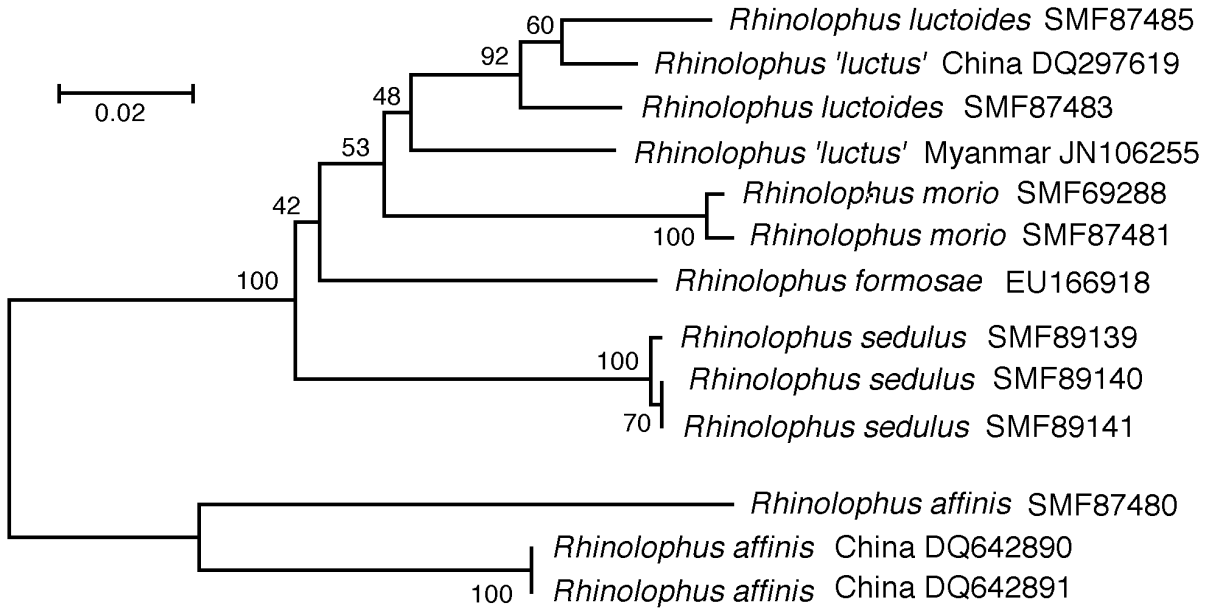

FIG. 11. Neighbour-joining tree based on 390 bp of the mitochondrial Control Region (D-loop). GenBank accession numbers for specimens studied here (indicated by SMF accession numbers) are given in Table 1. All specimens belonging to the *trifoliatus* group deposited in GenBank were included. *Rhinolophus affinis* was used as an out-group. Numbers at the branches refer to bootstrap support values

comprise rich material of *R. luctus* populations from the northern parts of the distributional range, and therefore external and cranial measurements can be found in several publications (Sinha, 1973; Topál and Csorba, 1992; Bates and Harrison, 1997; Bates *et al*., 2004; Soisook *et al*., 2010). In contrast, only few specimens have been collected in Indonesia and up to now a complete set of cranial measurements for the nominate subspecies from this region has not been published.

From the measurements normally used for skull description, we have chosen only three to describe the proportions of the skull. Two ratios were built by dividing lower toothrow length (CM_3L) and zygomatic width (ZW), respectively, by mandible length (ML). Values of these ratios are given in Table 3 for all members of the *trifoliatus* group. The data are graphically depicted in Fig. 12 (upper image). It can be seen that *R. luctoides* differs clearly from all other taxa concerning the CM_3L/ML ratio. *R. morio* however, showing a similar CM_3L/ML ratio as most of its relatives, can be distinguished by the ZW/ML ratio (mean 0.75) because *R. formosae*, *R. l. foetidus*, *R. l. lanosus*, *R. l. perniger* and *R. luctoides* have a ZW/ML ratio lower than 0.73. In this respect, *R. morio* shows greater similarity in skull proportions to other members of the *trifoliatus* group, i.e., *R. beddomei*, *R. sedulus* and *R. trifoliatus*, all clearly separate species. For that reason we propose to elevate the former subspecies *morio* to specific rank, *Rhinolophus morio* Gray, 1842. A second reason for recognizing *morio* as a discrete species is the unique Y-autosomal translocation.

As especially zygomatic width shows high intraspecific variability, it is advisable to calculate the mean value from as many specimens as possible. For that reason the high ZW/ML ratio (0.78) of the holotype of *R. luctus luctus* from Java should be confirmed by measurements of additional specimens.

The fact that these closely related species, formerly subspecies of *R. luctus*, differ so clearly in skull proportions is remarkable. A long toothrow and narrow zygomata as in *R. luctoides* are also found in many other *Rhinolophus* species from Southeast Asia (Fig. 12, lower image). A short toothrow as in *R. morio* but even broader zygomata are reported for Palaearctic and African rhinolophids. As was pointed out by Bogdanowicz (1992), different skull proportions might result in different prey preferences.

Further investigations will possibly show that not only in Peninsular Malaysia but also in other countries two closely related but genetically different '*R. luctus*' taxa exist. There are some reports of single specimens, which did not fit into the normal measurements for the respective locality. One male with forearm (FA) of 63 mm from Cambodia

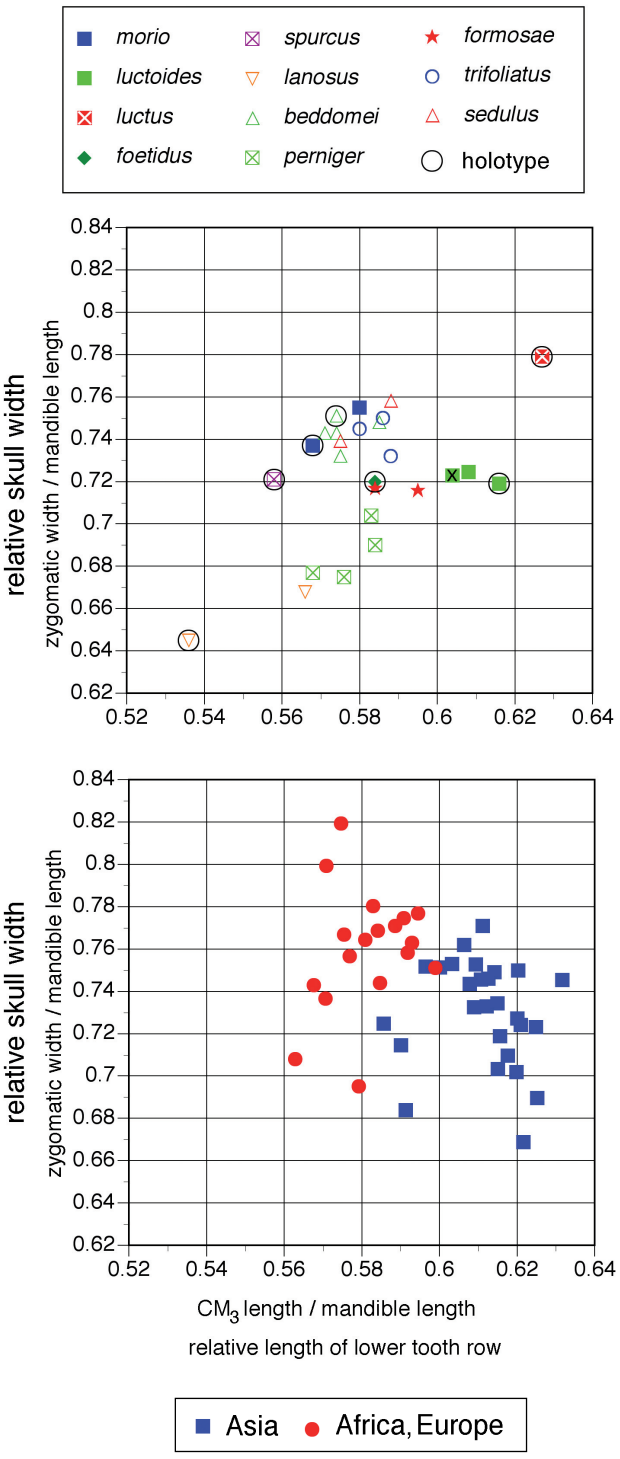

FIG. 12. Size-independent cranial ratios (relative length of lower toothrow and relative skull width) in taxa of the *trifoliatus* group (above) and of selected *Rhinolophus* species (below, data taken from Csorba *et al.*, 2003, without taxa depicted in the upper image). The ratios were calculated from mean values except for the respective holotypes, which are indicated by the taxon symbol surrounded by a circle. The specimen from Semangko Gap, Selangor (Topál and Csorba, 1992) which according to cranial ratios is presumably belonging to *R. luctoides* is indicated by a black cross on the *luctoides* symbol (green square)

(Hendrichsen *et al.*, 2001) and a male with FA 53 mm from southern Thailand (Soisook *et al.*, 2010) have been presumed to be similar to *R. beddomei*. However, concerning skull proportions, they appear to be quite different from *beddomei*. Recently, the specimen from Thailand has been assigned to the subspecies *thailandicus* of the newly described *Rhinolophus francisi* (Soisook *et al.*, 2015). Further, in a montane habitat in Java, a large female (FA 73 mm), the only specimen known of *R. l. geminus*, was collected which according to Andersen (1905) was more similar to *R. l. perniger* than to *R. l. luctus*. Topál and Csorba (1992) contributed novel insights into these issues. According to their analysis of 38 cranial and dental characters, they regarded the taxon *beddomei* as different from *R. luctus* at specific level. Their analysis also showed that while specimens from Northern Thailand clustered with members of the subspecies *R. l. perniger*, those from Central Thailand clustered with *R. l. foetidus* from Borneo and therefore were considered to belong to *R. l. morio*. Interestingly, the only Peninsular Malaysian specimen studied by Topál and Csorba (1992) was separated from the Thailand/Borneo cluster. As the sampling locality was reported as Semangko Gap (Fraser's Hill), which represents a montane habitat, and as the cranial proportions (CM_3L/ML 0.6; ZW/ML 0.72) fit well into the range of *R. luctoides* (Fig. 12, upper image), we suppose that this specimen could also belong to *R. luctoides*. However, for an unequivocal assignment the knowledge of the karyotype would be necessary.

In addition to the skull proportions, the size of the baculum can be used as discriminating feature for *R. luctoides* and *R. morio*. The baculum of *R. morio* (about 3 mm in length) is at least 1 mm smaller than that of *R. luctoides*. The only known baculum of an Indonesian specimen from Bali (which is possibly belonging to *R. l. luctus*) shows an intermediate size of 3.5 mm (Heller and Volleth, 1988). From other related taxa, only the bacula of two *R. l. perniger* specimens have been reported (Agrawal and Sinha, 1973). With a length of 6.7 and 7.0 mm, respectively, they are considerably larger than those of the Malaysian specimens.

Echolocation Frequency

The call frequencies of forms subsumed under *R. luctus* have been recorded from China in the north to Malaysia in the south. Two clearly distinct frequency ranges have been observed. Specimens from China, Laos and Thailand (FA 66–73 mm)

emitted calls from 32 to 34.9 kHz (Francis, 2008; Zhang *et al*., 2009; Soisook *et al*., 2010) whereas 40 to 42.6 kHz calls have been recorded from specimens with FA 63–65 mm in Peninsular Malaysia, Singapore and Sabah (Roberts, 1972; Kingston *et al*., 2000; Pottie *et al*., 2005; Francis, 2008; this study). The recordings of rather large specimens (FA more than 70 mm) from Thailand made with a QMC Mini Bat Detector resulting in a call frequency of 40 kHz (Robinson, 1996) clearly need confirmation.

Most of these observations reflect the well-known interspecific relationship of body-size and call frequency (e.g., Heller and von Helversen, 1989). Due to quite similar forearm length it seems unlikely that *R. morio* and *R. luctoides* could be distinguished by call frequency. If we assume that the reported frequency of 42.6 kHz (two specimens) from Singapore (Pottie *et al*., 2005) is representative for *R. morio* and the call frequency of 42 kHz of our female from Cameron Highlands is representative for *R. luctoides*, then indeed both species are indistinguishable in this respect.

In summary, our combined comparative genetic and morphological analyses support the elevation of *R. morio* to specific rank and the description of *R. luctoides* as novel species. As a consequence of these proposals the distribution of *R. luctus* would become discontinuous unless the subspecies in the northern parts of the distributional range would also receive a separate specific status. However, for determination of the taxonomic status of taxa hitherto recognised as subspecies of *R. luctus*, i.e., *perniger*, *lanosus*, *spurcus* and *foetidus*, the knowledge of the G-banded karyotype, supplemented by FISH data, and of DNA sequence divergence is urgently needed. In addition to such information, more morphological data are needed from Sumatra, Java and Bali for the nominate taxon of *R. luctus*.

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