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Karyotypic evolution in family Hipposideridae (Chiroptera, Mammalia) revealed by comparative chromosome painting, G- and C-banding

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Abstract: Comparing to its sister-family (Rhinolophidae), Hipposideridae was less studied by cytogenetic approaches. Only a few high-resolution G-banded karyotypes have been reported so far, and most of the conclusions on the karyotypic evolution in Hipposideridae were based on conventional Giemsa-staining. In this study, we applied comparative chromosome painting, a method of choice for genome-wide comparison at the molecular level, and G- and C-banding to establish comparative map between five hipposiderid species from China, using a whole set of chromosome-specific painting probes from one of them (*Aselliscus stoliczkanus*). G-band and C-band comparisons between homologous segments defined by chromosome painting revealed that Robertsonian translocations, paracentric inversions and heterochromatin addition could be the main mechanism of chromosome evolution in Hipposideridae. Comparative analysis of the conserved chromosomal segments among five hipposiderid species and outgroup species suggests that bi-armed chromosomes should be included into the ancestral karyotype of Hipposideridae, which was previously believed to be exclusively composed of acrocentric chromosomes.

Key words: Comparative chromosome painting; Robertsonian translocation; Paracentric inversion; Hipposideridae; Chiroptera

蹄蝠科的核型进化：比较染色体涂色、G 带和 C 带分析

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摘要：与其姐妹科(菊头蝠科)相比，蹄蝠科的细胞遗传学研究较少。迄今为止，仅少数蹄蝠科几个物种有高分辨率的 G 带核型报道，且有关该科核型进化的大多数结论都是基于常规 Giemsa 染色研究而得。该研究利用三叶小蹄蝠的染色体特异探针，通过比较染色体涂色、G 和 C 显带，建立了 5 种蹄蝠的染色体同源性图谱，并探讨了它们同源染色体间的 G 和 C 带异同。结果表明：罗伯逊易位、臂内倒位以及异染色质的扩增可能是蹄蝠科物种核型进化的主要机制。通过对这 5 种蹄蝠物种及其外群物种之间的同源染色体片段的比较分析，作者推测蹄蝠科的祖先核型并不像先前认为的全由端着丝粒染色体组成, 而应该含有中着丝粒染色体。

关键词：比较染色体涂色；罗伯逊易位；臂内倒位；蹄蝠科；翼手目
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The family Hipposideridae comprises nine extant genera and about 80 species (Simmons, 2005). Currently hipposiderid bats are found throughout temperate zone of the Old World from West Africa east to the New Hebrides and extend marginally into the Palaearctic (Corbet & Hill, 1991, 1992; Koopman, 1994). Based on the neontological data, Bogdanowicz & Owen (1998) suggested an Asian origin for the Hipposideridae. Although this family has been extensively studied during the past 150 years (summarized in Hill, 1963), its own phylogeny and phylogenetic relationship with its sister family, Rhinolophidae, remain controversial. Some researchers intended to treat Hipposideridae as a subfamily of Rhinolophidae (Ellerman & Morrison-Scott, 1966; Koopman, 1984, 1993, 1994; Simmons, 1998), while others suggested that Rhinolophidae and Hipposideridae should be treated as two different families (Corbet & Hill, 1992; Bogdanowicz & Owen, 1998; Hand & Kirsch, 1998; Nowak et al, 1999; Wang et al, 2003; Li et al, 2007). Likewise, morphological and molecular data supported that *Aselliscus* was a true hipposiderid and at the root of the *Hipposideros* lineage (Bogdanowicz & Owen, 1998; Hand & Kirsch, 1998), while immunological data indicated that *Aselliscus* was closer to *Rhinolophus* than to *Hipposideros* (Pierson, 1986). However, the most recent phylogenetic study of Hipposiderida has rejected both of the above hypotheses (Li et al, 2007).

To better understand the Hipposideridae phylogeny, other independent data, such as comparative cytogenetics, are needed. So far, 24 hipposiderid species have been karyotyped with six different diploid chromosome numbers (2*n*), 2*n*=30, 32, 36, 40, 50 and 52 (summarized in Bogdanowicz & Owen, 1998; Sreepada et al, 1993). However, some conclusions on the karyotypic evolution in Hipposideridae (Ando et al, 1980; Sreepada et al, 1993; Bogdanowicz & Owen, 1998; Wu & Harada, 2006) need to be further verified because of the low resolution of the conventional cytogenetic technique itself.

Cross-species chromosome painting has greatly improved the efficiency and accuracy of comparative cytogenetics of bats. This technique has already been successfully applied to the comparative cytogenetics of eight Chiropteran families (Volleth et al, 1999, 2002; Pieczarka et al, 2005; Ao et al, 2006, 2007; Mao et al, 2007, 2008). Phylogenetic analysis by outgroup comparison proved to be a useful tool for the study of karyotypic relationships and the mechanism of karyotypic evolution. Two entirely conserved homologous chromosomes were discovered only between *A. stoliczkanus* and *H. lavatus* (Ao et al, 2007), providing supports for the conclusion based on morphological and molecular data. In addition, the classification of Rhinolophidae and Hipposideridae as two different families was also supported by the molecular cytogenetic studies (Volleth et al, 2002; Ao et al, 2007). However, up to now only two species in Hipposideridae (*H. lavatus*, Volleth et al, 2002; Mao et al, 2007; *A. stoliczkanus*, Ao et al, 2007) have been studied by this technique using probes from human, *A. stoliczkanus*, and *Myotis myotis*, respectively.

Here, we reported the chromosome homologies of three other hipposiderid species from China using a whole set of chromosome-specific painting probes from *A. stoliczkanus*. Combining the data of G- and C-banding and previously published bats chromosome painting results, the karyotypic relationships and chromosomal evolution of Hipposideridae, especially, genus *Hipposideros*, were discussed.

1 Materials and Methods

1.1 Specimens

Five hipposiderid bats were collected from southwestern China (*H. larvatus*, 2 ♂, 1 ♀, Anlong, Guizhou; *H. armiger*, Canaliculus cave, 1 ♂, 2 ♀, Yiliang, Yunnan; *H. pratti*, 1 ♀, Emei, Sichuan; *H. pomona*, 1 ♂, 1 ♀, Guizhou; *A. stoliczkanus*, 1 ♂, Heshang cave, Kunming, Yunnan). The specimens have been deposited in the Museum of Vertebrates, Kunming Institute of Zoology, the Chinese Academy of Science (KIZ, CAS), P. R. China.

1.2 Cell culture, chromosomal preparation, G-banding and C-banding

Metaphase spreads were prepared from fibroblast cultures following standard protocols as described previously (Nie et al, 1998). The GTG- and C-banding were carried out according to conventional procedures.

1.3 Fluorescence *in-situ* hybridization (FISH)

The same set of chromosome-specific paints of *A. stoliczkanus* generated from flow-sorted chromosomes as described previously (Mao et al, 2007) were used in this study. Comparative chromosome painting between *A. stoliczkanus* and *Hipposideros* species followed the procedures described previously (Yang et al, 2000). For two-color FISH, biotin-labeled probes were visualized by Cy3-avidin (final concentration 1μg/mL, Amersham), FITC-labeled probes were detected with a layer of rabbit anti-FITC IgG (1∶500, Vector Laboratories) followed

by a layer of FITC-conjugated, goat anti-rabbit IgG (1∶250, Vector Laboratories).

1.4 Image capture and processing

Images were captured using the Genus System (Applied Imaging Corp, UK) with a CCD camera mounted on a Zeiss Axioplan 2 microscope as previously described in Yang et al (2000). Hybridization signals were assigned to specific chromosomes or chromosome regions defined by enhanced 4', 6-Diamidino-2-phenylindole (DAPI) banding patterns.

1.5 Chromosome nomenclatures

The G-banded chromosomes of three *Hipposideros* species (*H. armiger*, *H. pratti* and *H. pomona*) were arranged based on the previously reported karyotype of *H. lavatus* (Volleth et al, 2002; Mao et al, 2007). The C-banded chromosomes of five hipposiderid species were arranged roughly based on their correspondent G-banded karyotypes.

2 Results and Discussion

2.1 Highly conserved karyotypes in the genus *Hipposideros*

The G-banded karyotype of *H. pratti* (Fig. 1a) is reported for the first time, comprising 15 pairs of meta- and submetacentric autosomes, and one pair of medium-sized submetacentric X chromosomes. The G-banded karyotypes of *H. pomona* and *H. armiger* are similar to the previously reported ones (Sreepada et al, 1993; Qumsiyeh et al, 1988) (Fig. 1b,c). The G-banded karyotypes of *H. larvatus* and *A. stoliczkanus* have been described in our previous studies (Mao et al, 2007; Ao et al, 2007). All fourteen *A. stoliczkanus* autosomal paints and X chromosome paint were hybridized onto the metaphases of three *Hipposideros* species (*H. pomona*, *H. armiger* and *H. pratti*). Fluorescence *in-situ* hybridization (FISH) examples are shown in Fig. 2. The hybridization results are summarized onto the G-banded karyotypes of each species (Fig. 1). The *A. stoliczkanus* paints gave almost identical hybridization patterns on these three *Hipposideros* species. Ten of fourteen *A. stoliczkanus* autosomal paints (AST 1, 2, 4 - 8, 10, 13 and 14) each detected two homologous segments. Four paints (AST 3, 9, 11 and 12) each painted one pair of chromosomes. The probe of *A. stoliczkanus* X chromosome painted the whole X chromosome. In total, all the *A. stoliczkanus* autosomal paints detected 24 homologous segments in the genomes of *Hipposideros* species.

Hill (1963) classified the genus *Hipposideros* into seven species-groups and four of them have been represented in this study. At the level of conventional stained karyotypes, the genus *Hipposideros* showed considerable karyotypic conservatism, for all but one species, 2*n*=32 and *FN*=60 (summarized in Bogdanowicz & Owen, 1998). Consistent with this finding, the probes from *A. stoliczkanus* detected almost identical painting patterns in these four *Hipposideros* species. Additionally, the G-banding patterns of their homologous chromosomes were highly conserved (Fig. 3). Thus our results supported the hypothesis that the apparent karyotypic uniformity of the *Hipposideros* species reflected a true evolutionary conservatism (Sreepada et al, 1993).

2.2 Karyotypic relationships of the five hipposiderid species

Although *A. stoliczkanus* and four *Hipposideros* species belong to the same family (Nowak et al, 1999), extensive chromosome rearrangements (e.g. Robertsonian translocations) must have occurred between the present karyotypes of *A. stoliczkanus* and *Hipposideros* species. To convert the karyotype of *A. stoliczkanus* to that of *H. pomona*, 10 fissions and 9 fusions must be invoked (see Fig. 1 in Mao et al, 2007 and Fig. 1 in this study). In addition, a large number of paracentric inversions might also occur based on the results of chromosome painting and G-banding comparison. For example, one paracentric inversion (in the p arm of AST 11) must be invoked to convert the karyotype of *A. stoliczkanus* to that of *H. pomona*. Interestingly, another paracentric inversion had occurred on the same chromosome (AST 11) to form the corresponding chromosome of *H. larvatus*, *H. armiger* and *H. pratti* (Fig. 3). Finally, variations were also found in the sex chromosomes of *A. stoliczkanus* and *Hipposideros* species (e.g. the position of the centromere in the X-chromosome and the size of Y chromosome). *H. pomona* has almost identical X chromosome with *A. stoliczkanus*. However, a paracentric inversion is required to convert the X chromosome of *A. stoliczkanus* to that of *H. larvatus*, *H. armiger* and *H. pratti* (Fig. 3). The sizes of Y chromosomes are different among these four species with the largest one in *H. larvatus* and the smallest one in *H. pomona* (Fig. 3).

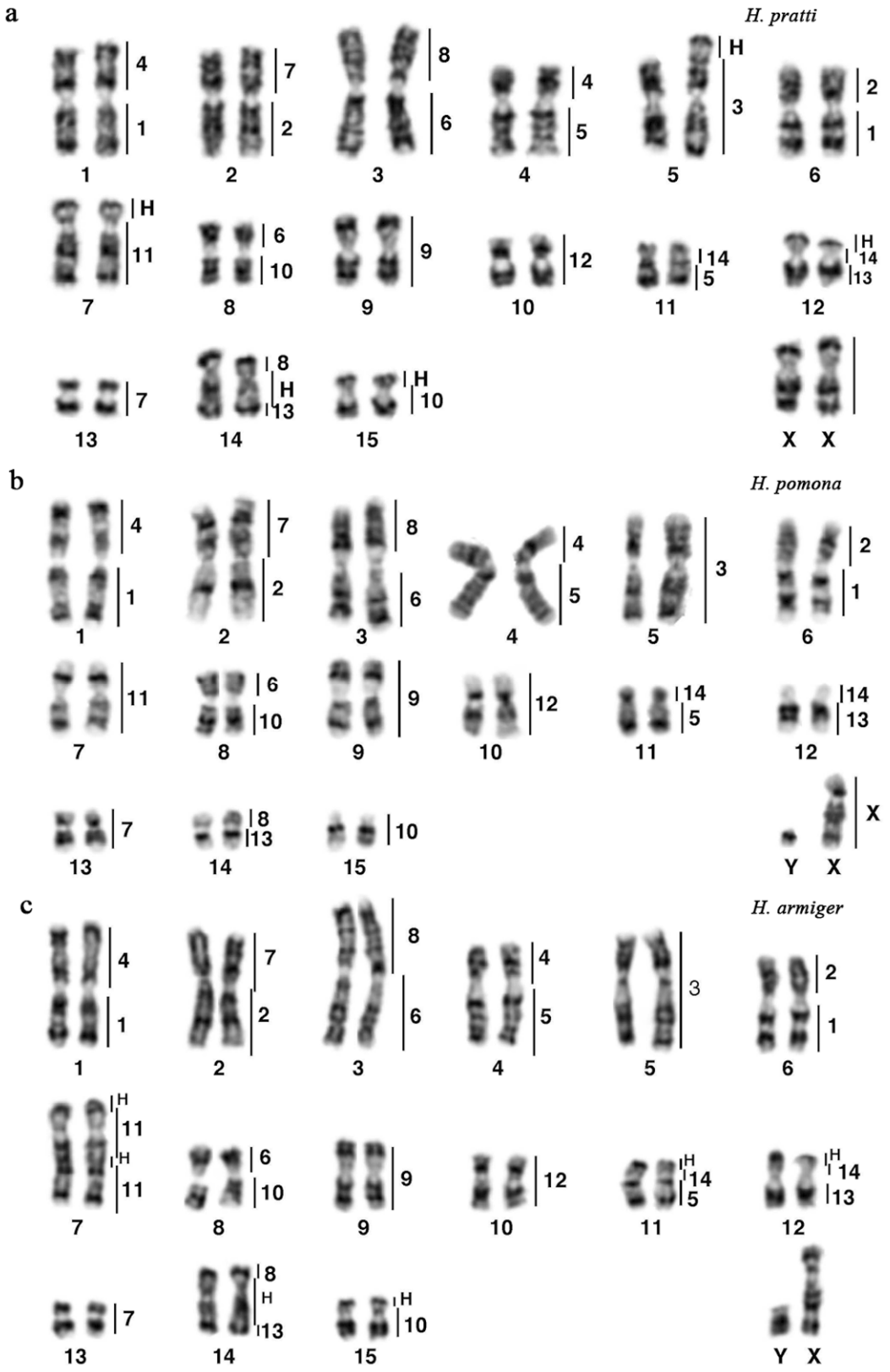

Fig. 1 Summary of the chromosome painting results with *A. stoliczkanus* paints onto G-banded karyotypes of *H. pratti* (a), *H. pomona* (b) and *H. armiger* (c)

Chromosome numbers are indicated below the chromosomes in each species and the segments homologous to *A. stoliczkanus* are indicated to the right of each chromosome. The capital letter "H" in some chromosomes indicates heterochromatin.

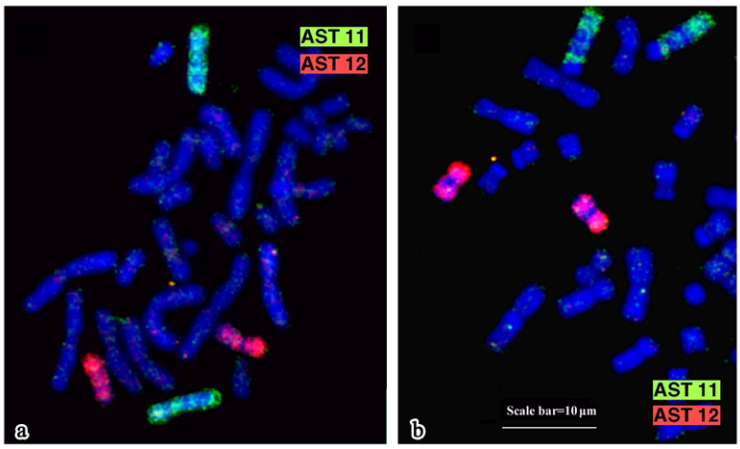

Fig. 2 FISH examples showing the hybridization patterns of *A. stoliczkanus* chromosome-specific paints onto metaphases of two *Hipposideros* species

Hybridization of AST 11 and 12 on chromosomes 7 and 10 of *H. pomona* (a), chromosomes 7 and 10 of *H. armiger* (b), respectively.

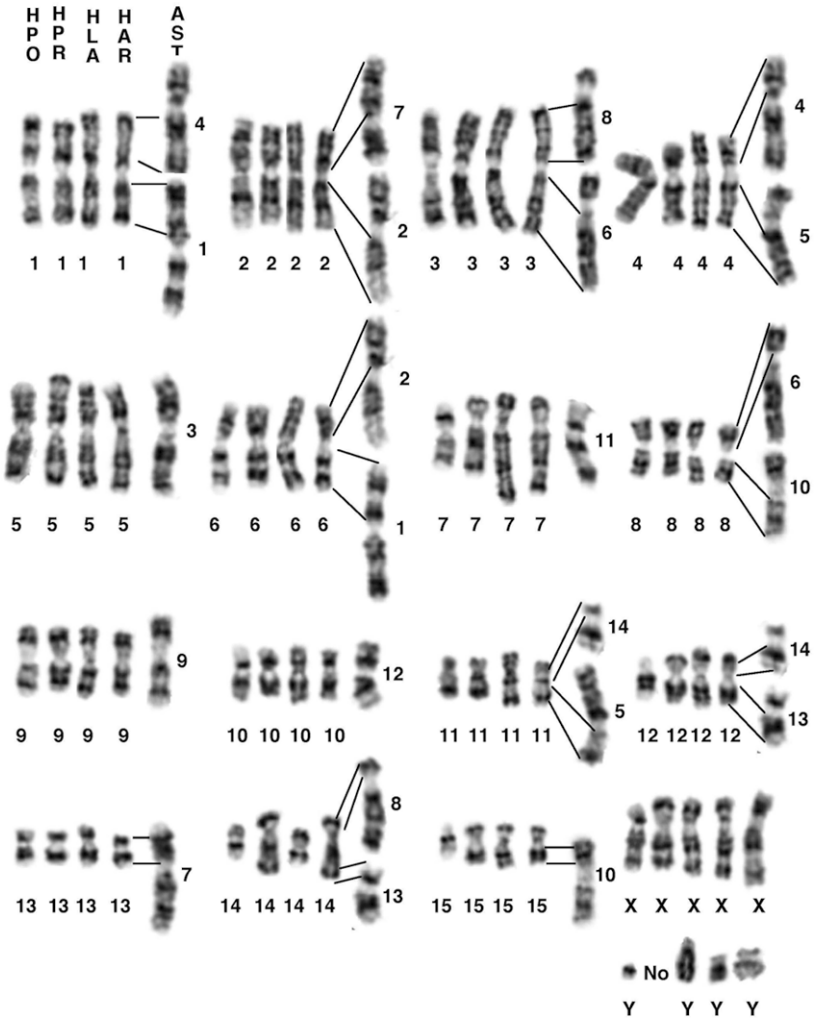

Fig. 3 Genome-wide chromosomal correspondence among five hipposiderid species revealed by *A. stoliczkanus* chromosome-specific painting probes

AST: *A. Stoliczkanus*; HPO: *H. pomona*; HPR: *H. pratti*; HLA: *H. larvatus*; HAR: *H. armiger*. Chromosome numbers are indicated below the chromosomes in each species and the segments homologous to *A. stoliczkanus* are indicated to the right of each chromosome. The capital letter "H" in some chromosomes indicates heterochromatin.

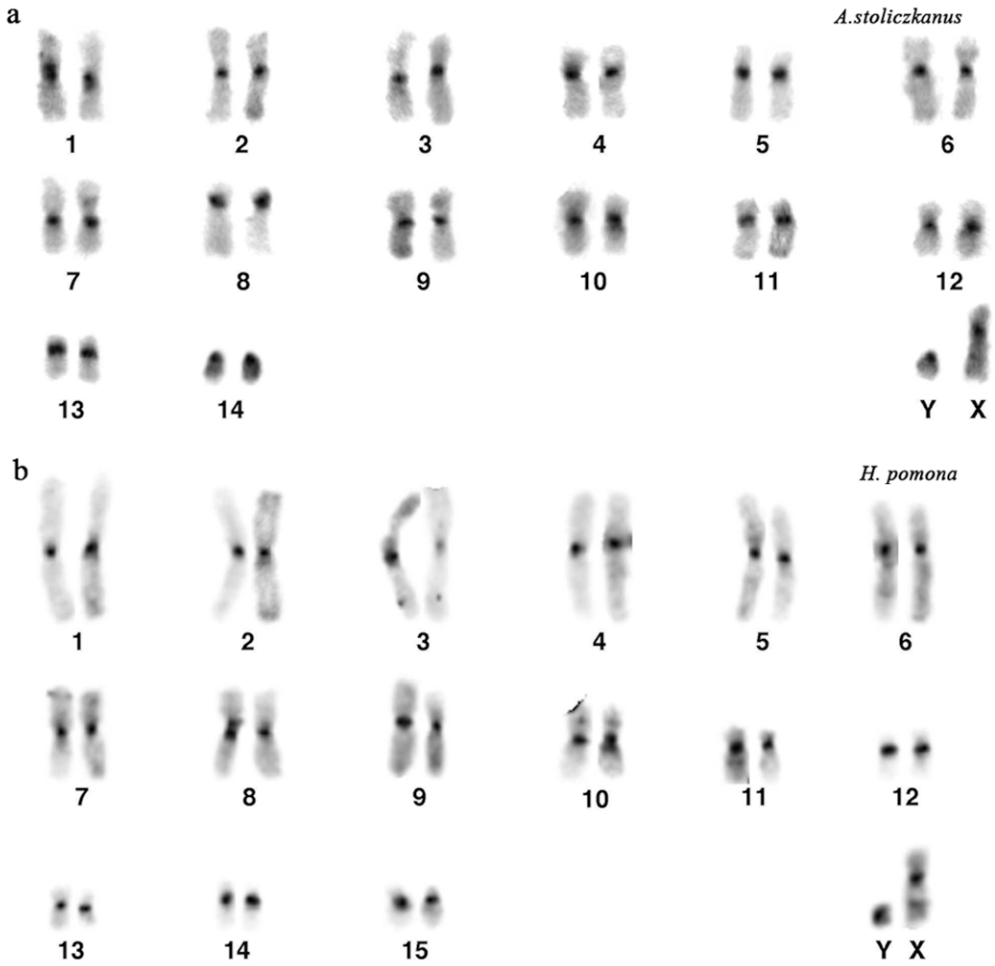

Fig. 4 C-banded karyotypes of *A. stoliczkanus* (a) and *H. pomona* (b)

Chromosome numbers are indicated below the chromosomes in each species.

In addition to paracentric inversions, the variation of the amount of heterochromatin may also play an important role in differentiating the karyotypes of *Hipposideros*. In *A. stoliczkanus* and *H. pomona*, C-band positive heterochromatic blocks are only found in the pericentromeric regions of autosomes (Fig. 4a,b). However, large blocks of heterochromatin have also been observed in the short arms of chromosomes 7, 11, 12 and 15 of *H. larvatus*, *H. armiger* and *H. pratti* (Fig. 5a, b, c). Additionally, *H. armiger* and *H. pratti* have an extra large intercalary heterochromatic block in chromosomes 14 (Fig. 5b,c). However, because of the limited specimens of each species used in this study, we can not rule out the possible involvement of polymorphism in the amount of heterochromatin observed in this study. Therefore, the data of heterochromatin should be treated cautiously when used in the phylogenetic study.

2.3 The ancestral chromosomal complements of Hipposideridae

Based on the conventional staining data, the ancestral karyotype of Hipposideridae was proposed to be composed of exclusively acrocentric chromosomes (Ando, 1980; Bogdanowicz & Owen, 1998). Combined analysis of the chromosome painting data of five hipposiderid species studied here and previously published data (Volleth et al, 2002; Mao et al, 2007) has provided new insight into the possible complements of the ancestral karyotype of Hipposideridae.

These five hipposiderid species shared two entire chromosomes (homologous to AST 12 and 13) (Fig, 3) which were also observed in the genomes of *R. leschenaulti* (Pteropodidae) and *M. altarium* (Vespertilionidae) (Mao et al, 2007). These two chromosomes could thus be regarded as the ancestral condition of Hipposideridae. Consistent with this suggestion, Volleth et al (2002) identified 25 different evolutionarily conserved units (ECUs), among which

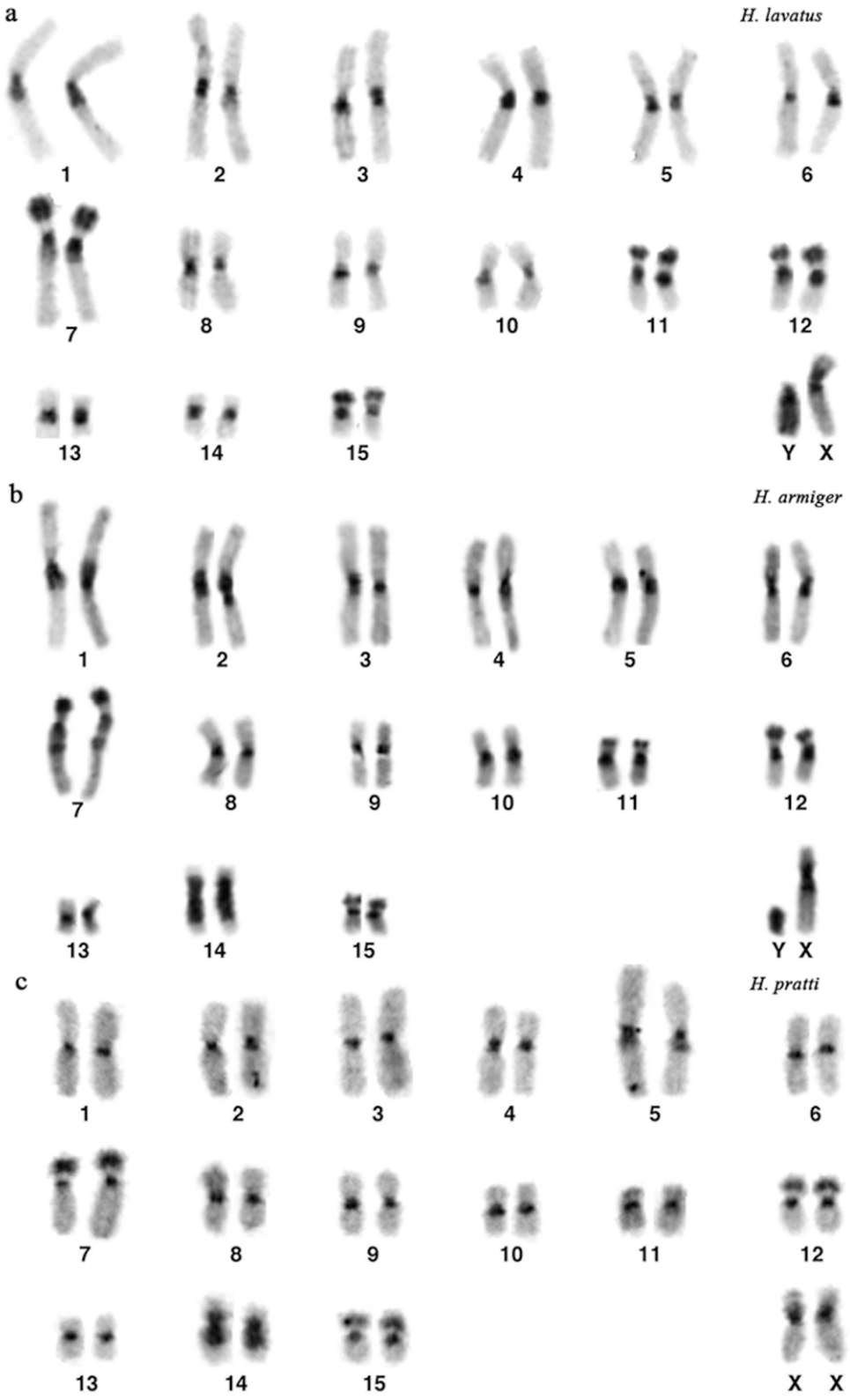

Fig. 5 C-banded karyotypes of *H. larvatus* (a), *H. armiger* (b) and *H. pratti* (c)
Chromosome numbers are indicated below the chromosomes in each species.

ECU 11a and ECU 11b-22b-12b, ECU 18:20, and ECU 7a (equivalents of AST 11, 12, 13) (Mao et al, 2007) were considered as ancestral segments of Chiroptera. Thus, our comparative chromosome maps and outgroup

comparison suggested that the bi-armed chromosomes (e.g. equivalents of AST 2 and 13) should be included in the ancestral karyotype of Hipposideridae. In the future more karyotypes of species in Hipposideridae will be necessary to access the exact number of chromosomes in the ancestral Hipposideridae karyotype.

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