Karyotypic evolution of *Apodemus* (Muridae, Rodentia) inferred from comparative FISH analyses

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Summary

We conducted comparative FISH analyses to investigate the chromosomal rearrangements that have occurred during the evolution of the rodent genus *Apodemus*, which inhabits broadleaf forests in the temperate zone of the Palaearctic region. Chromosome-specific painting probes of the laboratory mouse were hybridized to chromosomes of seven *Apodemus* species, *A. agrarius*, *A. argenteus*, *A. gurkha*, *A. peninsulae*, *A. semotus*, *A. speciosus* and *A. sylvaticus*, and homologous chromosomal regions were determined in the species for the study of karyotypic evolution. Differences in the hybridization patterns were found in nine pairs of autosomes among the seven species. The chromosomal location of the 5S rRNA genes on the telomeric region of chromosome 20 was highly conserved in all the species. In contrast, there was much wider variation in the location of the 18S-28S rRNA genes, although the 18S-28S rRNA genes were predominantly located on chromosomes 7, 8 and 12. Phylogenetic relationships of the seven *Apodemus* species were inferred from the chromosome rearrangements and the chromosomal distribution patterns of the 18S-28S rRNA genes. The karyotypic relationships correlated well with the molecular phylogeny, and *A. semotus* had the most highly conserved karyotype among the seven species.

Keyword: *Apodemus*, FISH, ribosomal RNA genes, karyotypic evolution, phylogeny.

Introduction

Small rodent species belonging to the genus *Apodemus* are distributed throughout the Palaearctic region (Corbet 1978, Corbet & Hill 1992, Orlov *et al.* 1996), and more than 20 species are now recognized in this genus (Musser & Carleton 1993). Since all the extant species live in broadleaf forests of the temperate zone, the characteristics of habitat preferences have been inherited from their ancestors, and the radiation of the species might have been associated with global environmental changes, especially changes of flora (Serizawa *et al.* 2000).

There have been several studies on the phylogenetic relationships of the *Apodemus* species based on molecular data such as restriction fragment length polymorphism of the ribosomal RNA genes and the nucleotide sequences of the mitochondrial and nuclear genes (Suzuki *et al.* 1990, Chelomina *et al.* 1998, Serizawa *et al.* 2000, Michaux *et al.* 2002). Serizawa *et al.* (2000) constructed molecular phylogenetic trees of nine *Apodemus* species with the nucleotide sequences of the cytochrome *b* (*Cytb*) gene and the interphotoreceptor retinoid binding protein (*IRBP*) gene, and grouped them into four different lineages: the *Agrarius* group (*A. agrarius*, *A. peninsulae*, *A. semotus* and *A. speciosus*), *Argenteus* group (*A. argenteus*), *Gurkha* group (*A. gurkha*) and *Sylvaticus* group (*A. alpicola*, *A. flavicollis* and *A. sylvaticus*).

Comparative studies of the karyotypes in this genus have been performed using chromosomal banding techniques (Soldatovic *et al.* 1975, Yoshida *et al*, 1975, Vujosevic *et al.* 1984, Saitoh & Obara 1986, Orlov *et al.* 1996, Reutter *et al.* 2001). The diploid chromosome number of most *Apodemus* species is 2n = 46 - 48, and their fundamental number (FN) ranges from 48 to 56. The diversity of karyotypes has been achieved by the accumulation of chromosome rearrangements that occurred during evolution, and thus it has been possible to define the history of karyotypic changes by comparing the karyotypes among the extant species. Chromosome banding is an effective method for demonstrating

chromosome homologies between phylogenetically related species, but the resolution using this approach is not as high as that of analysis at the molecular level.

Comparative chromosome painting is a robust method for detecting chromosome homologies at the whole chromosome level, and this approach provides clues about the processes of karyotypic evolution (Wienberg *et al*. 1990, Scherthan *et al*. 1994, Wienberg & Stanyon 1995). The 5S and 18S-28S ribosomal RNA (rRNA) genes are good cytogenetic markers to examine inter- and intraspecies chromosomal variations, and in our previous study, we defined the chromosome rearrangements between the laboratory mouse (*Mus musculus*) and the Indian spiny mouse (*M. platythrix*) by comparative chromosome painting and mapping of the rRNA genes (Matsubara *et al*. 2003). The main event of the chromosome rearrangements between the two species was tandem fusion that occurred in the ancestral karyotype of the *Mus* species, and fission, reciprocal translocations and insertions were also involved.

In this study, to clarify the processes of the karyotypic changes during the evolution of seven *Apodemus* species, we conducted comparative chromosome painting with chromosome-specific painting probes of the laboratory mouse. The chromosomal locations of the 5S and 18S-28S rRNA genes were also determined in the seven *Apodemus* species. The karyological data were compared with the molecular phylogenetic data, and karyotypic evolution in *Apodemus* is discussed.

Materials and methods

Animals, chromosome preparation and FISH

The species used in this study and the localities where they were originally collected are listed in Table 1. Agr1, Agr2, Sem1 and all animals of *A. sylvaticus* were bred at the Laboratory of Wild Animals, Department of Animal Sciences, Faculty of Agriculture, Tokyo University of Agriculture, Japan, and other animals

were originally captured in the field.

Preparation of R-banded chromosomes and FISH were performed as described by Matsuda *et al.* (1992) and Matsuda & Chapman (1995). Chromosome preparations were made from splenocytes or fibroblasts taken from lung tissue. The cultured cells were treated with BrdU during late S phase for differential replication banding. R-banded chromosomes were obtained by exposure of chromosome slides to UV light after staining with Hoechst 33258. For G-banding analysis, chromosome preparations were made from splenocytes or fibroblasts cultured without BrdU-treatment. The G-banded chromosomes were obtained with the GTG (G-bands by trypsin using Giemsa) method as described by Seabright (1971).

A 1.8-kb and a 6.6-kb mouse genomic DNA fragment were used for chromosomal localization of the 5S and 18S-28S rRNA genes, respectively (Kominami *et al.* 1982, Matsuda *et al.* 1994). The Tu89 clone used for mapping the *D17Leh89* locus was a cosmid DNA clone isolated from mouse chromosome 17 by microdissection (Búcan *et al.* 1987).

The biotinylated chromosome-specific painting probes of the laboratory mouse were purchased from Cambio Ltd., Cambridge, UK. *In situ* hybridization with the painting probes was carried out following our previously reported method (Matsubara *et al.* 2003) with slight modification. The chromosome slides were hardened for 2 h at 65˚C and denatured for 2 min at 70˚C in 70% formamide, 2 X SSC. The probes were denatured for 10 min at 75˚C and pre-annealed by incubation at 37˚C for 45 min. The chromosome slides were hybridized with the mouse painting probes for 3 or 4 days at 37˚C. The hybridized probes were reacted with FITC-avidin (Roche Diagnostics), and then stained with propidium iodide (PI).

Microphotography and image capture

The chromosomal slides were observed under Nikon fluorescence

microscope using Nikon filter sets B-2A and UV-2A. Kodak Ektachrome ASA100 films were used for microphotography. The digital FISH images were captured with the 550CW-QFISH application program of Leica Microsystems Imaging Solution Ltd. (Cambridge, UK) using a cooled CCD camera (MicroMAX 782Y, Princeton Instruments) mounted on a Leica DMRA microscope.

Results and discussion

G-banding

The G-banded karyotypes of the seven *Apodemus* species are shown in Figure 1. The karyotypes have been reported for all of these species except for *A. gurkha* (e.g., Soldatovic *et al.* 1975, Tsuchiya 1979, Vujosevic *et al.* 1984, Saitoh & Obara 1986, Obara *et al.* 1997). The diploid chromosome numbers and fundamental numbers found in this study were the same as those reported previously, except regarding the B chromosomes. Polymorphism was observed in the biarmed chromosome 20 of Arg1, which resulted from a pericentric inversion in acrocentric chromosome 20 or amplification of heterochromatin on the short arm (data not shown). The G-banded patterns in chromosomes larger than chromosome 16 were similar among the species, although submetacentric chromosome 16 of *A. agrarius* and submetacentric chromosomes 10 and 16 of *A. speciosus* were morphologically different from the chromosomes of other species. The remaining chromosomes smaller than chromosome 17 were too small to be reliably compared by banding analysis, and their banding patterns were so different among the species that chromosome homologies could not be morphologically identified.

Chromosome homology among Apodemus *species*

All mouse chromosome-specific probes except for the Y chromosome probe

successfully hybridized to chromosomes of all the *Apodemus* species (Figure 2). Three B chromosomes in *A. peninsulae* (Pen1) were not hybridized with any MMU (*Mus musculus*) probes (data not shown). The patterns painted with the mouse probes are summarized in Figure 1. Chromosomes of *A. peninsulae* were initially numbered according to their length since all the chromosomes were acrocentric. Each chromosome of the other six species was then numbered following the karyotype of *A. peninsulae*. The hybridization patterns were identical in 14 pairs of autosomes among the seven species. Evidence of structural changes was found in nine pairs of autosomes, chromosomes 2, 10, 16, 17, 19, 20, 21, 22 and 23. Both chromosomes 21 and 23 were painted by MMU 10 and 17. The *D17Leh89* locus located on mouse chromosome 17 (Búcan *et al*. 1987) was mapped as a cytogenetic marker to discriminate between chromosomes 21 and 23: the chromosome that carried it was classified as chromosome 21 (Figure 1).

The proximal region of chromosome 2 was hybridized with the MMU5 probe in six species, while this region was not painted in *A. argenteus* (Figures 1 & 2a). Chromosomes 11 and 22 were also hybridized with the MMU5 probe in seven species, and the size of the painted segment was almost the same among the species. These results suggest that the proximal region of chromosome 2 may have low gene density and so the nucleotide sequences have diversified in this chromosomal region of *A. argenteus* so that the hybridization intensity was too weak to detect the homologous segments with the MMU5 probe. A small segment homologous to MMU17 was detected in the proximal region of chromosome 17 in *A. argenteus*, while no hybridization signals were detected in six other species. A translocation might have occurred in this region of *A. argenteus* between chromosome 17 and chromosome 6 or 10.

Chromosome 10 was submetacentric in *A. speciosus*, and acrocentric in the six remaining species. Chromosome 16 was submetacentric in *A. agrarius* and *A. speciosus*, and acrocentric in the five remaining species. Chromosome 19 was

metacentric in *A. agrarius* and *A. gurkha*, and acrocentric in the five other species. Chromosome 20 was metacentric in *A. agrarius,* and acrocentric in the six other species. Chromosomes 21 and 22 were fused at the centromere in *A. argenteus* by Robertsonian translocation, and chromosome 22 was metacentric in *A. speciosus,* and acrocentric in the five other species. Chromosome 23 was acrocentric in *A. peninsulae* and *A. sylvaticus*, and metacentric in the five other species. However, the painting pattern was not identical in the acrocentric chromosome 23 between *A. peninsulae* and *A. sylvaticus* (Figures 1a, 1g, 2c & 2d). In our study, the only available European species was *A. sylvaticus*. The morphology and the G-banding patterns of autosomes are similar among at least four European species, *A. sylvaticus*, *A. flavicollis*, *A. alpicola* and *A. microps* (Vujosevic *et al.* 1984, Reutter *et al.* 2001). In contrast, accumulation of several chromosome rearrangements has occurred in the lineage leading to *A. agrarius* and *A. speciosus* (Figure 1b & d). Our cytogenetic data are in good accordance with the finding of Serizawa *et al.* (2000) that Asian species diverged earlier than European species diverged.

Chromosomal locations of the ribosomal RNA genes

Intraspecific variations were not observed in the chromosomal locations of the 5S and 18S-28S rRNA genes, except that the genes were located on B chromosomes in *A. agrarius* and *A. peninsulae* (see next section). The 5S rRNA genes were localized to the telomeric region of chromosome 20 in all the species except for *A. agrarius,* in which the genes were localized to the centromeric regions of the X and Y chromosomes and one B chromosome besides chromosome 20 (Table 2 & Figure 3a). The large hybridization signals on the X and Y chromosomes in *A. agrarius* suggest the possibility that the nucleotide sequence, which is homologous to the repetitive sequence in the centromeric heterochromatin of the sex chromosomes, may be contained in the spacer regions of the 5S rRNA genes, as reported in *Mus* species (Matsuda & Chapman 1995,

Matsubara *et al.* 2003). In our previous studies, we demonstrated that the location of the 5S rRNA genes on the distal telomeric region of chromosome 8 is evolutionarily conserved among *Mus* species (Matsuda *et al.* 1994, Matsubara *et al.* 2003). Chromosome 20 of the *Apodemus* species was also painted with MMU 8 probes, indicating that the location of the 5S rRNA genes is highly conserved in *Apodemus* species as well as *Mus* species.

The chromosomal locations of the 18S-28S rRNA genes were widely different among the species (Table 2 & Figures 3b - d). The distribution patterns of the 18S-28S rRNA genes in the seven species were well correlated with the phylogenetic relationships determined by Serizawa *et al.* (2000). In the *Agrarius* group and the *Argenteus* group of East Asia, the locations of the 18S-28S rRNA genes were relatively well conserved; the genes were predominantly located on chromosomes 7 and 8 with slight variation that was peculiar to each species (Table 2 & Figure 3b). On the other hand, the genes were localized to numerous chromosomes in *A. gurkha* in Nepal and *A. sylvaticus* in the western part of the Palaearctic region (Table 2, Figures 3c & d). The extensive distribution of the silver-stained nucleolus organizer regions (NORs) has also been reported in some European *Apodemus* species, including *A. sylvaticus* (Boeskorov *et al.* 1995). The locations of the 18S-28S rRNA genes appear to have been dispersed to numerous chromosomes by translocation or transposition between different chromosomes in these species. Such dispersion of the 18S-28S rRNA genes has been also reported in *Mus musculus* (Suzuki *et al.* 1990, Matsuda & Chapman 1995) and the hominoid (Hirai *et al.* 1999). The 18S-28S rRNA genes originally located on chromosomes 7 and 8 (and possibly 12) in the ancestral karyotype of the *Apodemus*, and these genes located on chromosomes 1 - 6 and 14 and chromosomes 11, 15, 16, 21, X and Y were seen specifically in *A. gurkha* and *A. sylvaticus*, respectively. These results suggest that these genes were independently dispersed in each lineage of *A. gurkha* and *A. sylvaticus* after divergence from the *Agrarius* and *Argenteus* groups.

B chromosomes

The presence of B chromosomes (Bs), which are supernumerary chromosomes other than the standard components (autosomes and sex chromosomes), has been recognized in several *Apodemus* species (Hayata 1973, Bekasova *et al*. 1980, Vujosevic & Zivkovic 1987, Kartavtseva 1994, Obara & Sasaki 1997, Zima *et al*. 1997). In this study, Bs were observed in all five individuals of *A. peninsulae* and one out of the three individuals of *A. agrarius* (Table 3). The Bs are generally considered to have originated from the autosomes and/or sex chromosomes, and to have undergone amplification of the B-specific repetitive DNA sequences (reviewed by Camacho *et al*. 2000). As supporting evidence for this idea, two types of repetitive DNA sequences, sequences common to both the Bs and autosomes and/or sex chromosomes, and B-specific sequences, have been obtained from several organisms. Recently, two different types of B components, B1 and B2, were obtained from *A. peninsulae* by microdissection (Trifonov *et al*. 2002). B1 chromatin contained DNA sequences that were homologous to the dispersed type of repetitive sequences of the standard components and that hybridized to most of the large Bs. On the other hand, B2 chromatin predominantly hybridized to the dot-like and small acrocentric Bs but not to the standard components. Those authors proposed that the two types of Bs originated independently in *A. peninsulae*. The 18S-28S rRNA genes were located on some Bs in all five individuals of *A. peninsulae* (Table 3 & Figure 3b). The location of the 18S-28S rRNA genes on Bs was also reported by Boeskorov *et al*. (1995), who found that the Ag-stained NOR regions were localized to two metacentric Bs in an individual captured in the Ussuri Nature Reserve, Russia. The 18S-28S rRNA genes appeared to be localized to meta- or submetacentric Bs but not to dot-like and small acrocentric Bs in this study (Table 3). The results of Trifonov *et al*. (2002) and our study confirm that the Bs have multiple origins in this species. The 5S rRNA genes were also

located on a B in one out of three individuals of *A. agrarius* (Agr3) (Table 3 & Figure 3a). The hybridization signal painted on the B indicates that this B was probably derived from centromeric heterochromatin of either an X or Y chromosome with 5S rRNA genes in this species.

There are wide differences in the number of Bs and the frequency of individuals with Bs among *Apodemus* species, and the presence of Bs has not been found in some species (Bekasova *et al*. 1980, Vujosevic & Zivkovic 1987, Kartavtseva 1994, Obara & Sasaki 1997, Zima *et al*. 1997). Therefore, the origins of Bs differ between individuals as well as between species, although the possibility that the 5S or 18S-28S rRNA genes transpose to some Bs from the standard components after the appearance of Bs has not been excluded. The mechanism of the appearance of Bs is still unknown and remains to be investigated in further studies.

Karyotypic evolution of genus Apodemus

We inferred the phylogenetic relationships of the seven *Apodemus* species from the chromosome rearrangements and the chromosomal distribution of the 18S-28S rRNA genes. Figure 4 shows the scheme of the karyotypic evolution in the seven species of *Apodemus,* assuming the most parsimonious events of chromosome rearrangements and translocations of the 18S-28S rRNA genes. The karyological relationships of the seven *Apodemus* species correspond well with the molecular phylogenetic tree constructed by Serizawa *et al*. (2000) based on the nucleotide sequences of the *IRBP* genes. To account for the process of the karyotypic evolution, we postulate two species that have not been found yet or do not exist at present. According to Serizawa *et al*. (2000), the four groups diverged within a short period (8 - 10 million years ago), and thus this type of divergence is regarded as adaptive radiation. It is therefore likely that the four groups diverged from one or only a few common ancestors. We speculate that the ancestral karyotype of *A. argenteus, A. gurkha* and *A. sylvaticus* was similar to

that of *A. semotus*, i.e. 22 pairs of acrocentric chromosomes, one pair of metacentric chromosomes and sex chromosomes. The 18S-28S rRNA genes are located on chromosomes 7, 8 and 12 in the former three species, whereas they are not located on chromosome 12 in *A. semotus*. The two types of karyotypes, in which the genes were located on chromosomes 7 and 8 or chromosomes 7, 8 and 12, were probably present as intraspecific variations in the ancestral species. In *A. argenteus, A. gurkha* and *A. sylvaticus,* the karyotype of each species might have originated from one chromosome rearrangement that occurred in the ancestral karyotype. The karyotype of *A. peninsulae* might have derived from the occurrence of one inversion followed by the appearance of Bs. It is possible that inversions in chromosomes 10 and 22 and inversions in chromosomes 19 and 20 could have occurred in the unknown species, which was derived from *A. semotus* by one inversion event in chromosome 16, and consequently led to the karyotypes of *A. speciosus* and *A. agrarius,* respectively.

Our karyological finding that *A. semotus*, an endemic species of Taiwan, retains the ancestral karyotype confirms the suggestion of Serizawa *et al.* (2000) that the ancestor of the extant species appeared in the forests of Central East Asia at late Tertiary. It is very likely that the ancestor of *A. semotus* was originally distributed in not only Taiwan but also in the continent and was superseded by new *Agrarius*-group species with extinction or reduction of the ancestors. Several authors have classified *A. argenteus* solely into a single group based on molecular, biochemical and morphological data (Musser *et al.* 1996, Serizawa *et al.* 2000). Several structural changes of chromosomes that were not found in six other species were observed in *A. argenteus* in this study. Thus, the ancestor of *A. argenteus* might have diverged much earlier than other species. The 18S-28S rRNA genes are distributed on many chromosomes in *A. gurkha* and *A. sylvaticus*, and the chromosomes with these genes are considerably different between the two species, except for chromosomes 7, 8, 12 and 22. These results suggest that the two species diverged from the common ancestor during a short period and the

genes rapidly dispersed to numerous chromosomes. However, very little molecular cytogenetic data has been obtained for European *Apodemus* species, and thus further studies of the species that inhabit the western part of the Palaearctic region will be required to understand the history of their karyotypic evolution.

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Figure legends

Figure 1. G-banded karyotypes of the seven *Apodemus* species with comparative cytogenetic maps between the *Apodemus* species: *A. peninsulae* (a), *A. agrarius* (b), *A. semotus* (c), *A. speciosus* (d), *A. argenteus* (e), *A. gurkha* (f) and *A. sylvaticus* (g), and the laboratory mouse. There have been no reports of detailed standard karyotypes of *Apodemus* species, and therefore chromosomes of *A. peninsulae* were numbered according to their length, and chromosomes of the six other species were numbered according to their homology to *A. peninsulae* chromosomes. The numbers of mouse chromosomes homologous to *Apodemus* chromosomes are indicated to the right of the chromosomes of each species. Arrows and arrowheads indicate the regions to which the following genomic clones were hybridized: the *D17Leh89* loci (black arrows), the 5S rRNA genes (white arrowheads), and the 18S-28S rRNA genes (black arrowheads).

Figure 2. FISH patterns obtained with mouse chromosome-specific painting probes. (a, b, d) Chromosome painting with the MMU 5 probe in *A. peninsulae* (Pen1) (a), and MMU 17 probe in *A. peninsulae* (Pen1) (c) and *A. sylvaticus* (Syl1) (d). Hybridization signals were visualized using FITC-avidin on R-banded chromosomes stained with PI. (b) Hoechst-stained G-banded pattern of the same metaphase of (a). (a) Arrows indicate the hybridization signals with MMU 5 on the proximal regions of chromosome 2. (c, d) Arrows indicate that the hybridization patterns with MMU 17 on chromosome 23 are different between *A. peninsulae* (c) and *A. sylvaticus* (d). Scale bars indicate 10 μm.

Figure 3. FISH patterns with genomic DNA clones of the 5S (a) and 18S-28S (b - d) rRNA genes. Chromosomal localization of the 5S rRNA genes in *A. agrarius* (Agr3) (a), and the 18S-28S rRNA genes in *A. peninsulae* (Pen2) (b), *A. sylvaticus* (Syl1) (c) and *A. gurkha* (Gur1) (d). Hybridization signals were visualized by FITC-avidin on PI-stained chromosomes. Arrowheads indicate the

hybridization signals on autosomes and sex chromosomes. (a, b) Arrows indicate the hybridization signals of the 5S (a) and 18S-28S (b) rRNA genes on B chromosome(s). Scale bars indicate 10 μm.

Figure 4. Phylogenetic relationships of the seven *Apodemus* species inferred from the chromosome rearrangements and the chromosomal distribution of 18S-28S rRNA genes. The chromosome numbers under each species name indicate the chromosomes to which the 18S-28S rRNA genes were localized. The type of chromosome rearrangements that occurred between two species is indicated on the side of each branch. In and Rb indicate inversion and Robertsonian translocation, respectively.

Table 1. Species used in this study, their diploid chromosome numbers (2n) and fundamental numbers (FN) .

Species	Common name	2n	FN excluding B(s)	Specimen[b]	Collection locality
A. agrarius	Striped field mouse	48 + B(s)	56	Agr1 (♀)	Korea
				Agr2 (♂)	Korea
				Agr3 (♀)	Vladivostok, Russia
A. peninsulae	Korean field mouse	48 + B(s)	48	Pen1 (♂)	Ussrisk, Russia
				Pen2 (♂)	Hokkaido, Japan
				Pen3 (♂)	Vladivostok, Russia
				Pen4 (♂)	Vladivostok, Russia
				Pen5 (♂)	Khasan, Russia
A. semotus	Taiwanese field mouse	48	50	Sem1 (♂)	Taiwan
				Sem2 (♂)	Taiwan
A. speciosus	Large Japanese field mouse	46 or 48[a]	56	Spe1 (♀)	Ibaraki, Japan
				Spe2 (♂)	Miyazaki, Japan
				Spe3 (♀)	Hokkaido, Japan
				Spe4 (♀)	Shizuoka, Japan
A. argenteus	Small Japanese field mouse	46 + B(s)	51 (♂); 52 (♀)	Arg1 (♂)	Miyazaki, Japan
				Arg2 (♂)	Kagawa, Japan
				Arg3 (♂)	Shizuoka, Japan
				Arg4 (♀)	Yakushima, Japan
A. gurkha	Himalayan field mouse	48	52	Gur1 (♂)	Nepal
A. sylvaticus	Wood mouse	48 + B(s)	48	Syl1 (♂)	Switzerland
				Syl2 (♂)	Germany
				Syl3 (♀)	Belgium

[a] Two chromosomal races exist (Tsuchiya *et al.* 1973).

[b] The individual with the first I.D. number was used for both chromosome painting and chromosome mapping of the 5S and 18S-28S rRNA genes in each species. Other individuals were only used for chromosome mapping of the rRNA genes.

Table 2. Chromosomal distribution of the 5S and 18S-28S rRNA genes.

Group[a]	Species	5S rRNA[b]	18S-28S rRNA[b]
Agrarius	*A. agrarius*	20, X, Y, B	7, 8, 22
	A. peninsulae	20	7, 8, B(s)
	A. semotus	20	7, 8
	A. speciosus	20	7
Argenteus	*A. argenteus*	20	7, 8, 12
Gurkha	*A. gurkha*	20	1, 2[c], 3, 4, 5, 6, 7, 8, 12, 14[c], 22
Sylvaticus	*A. sylvaticus*	20	7, 8, 11, 12, 15, 16, 21, 22, X, Y

[a] Grouping of species was made according to Serizawa *et al.* (2000).

[b] The chromosomal regions at which the genes are located are shown in Figure 1.

[c] The genes are located in a nonhomogeneous manner.

Table 3. Numbers of B chromosomes and the morphologies of B chromosomes with the 5S or 18S-28S rRNA genes in five *A. peninsulae* individuals and one *A. agrarius* individual.

Species	Specimen	No. of B(s)	Classification*		
			5S rRNA	18S-28S rRNA	No rRNA
A. peninsulae	Pen1	3		Medium m/sm (1), Small m/sm (1)	d (1)
	Pen2	7		Medium m/sm (4)	Small a (3)
	Pen3	2		Small m/sm (1), d (1)	
	Pen4	4		Medium m/sm (2), Small m/sm (2)	
	Pen5	1		Large m (1)	
A. agrarius	Agr3	1	d (1)		

m, metacentric chromosome; m/sm, meta- or submetacentric chromosome; a, acrocentric chromosome; d, dot-like chromosome

* The numbers of chromosomes are shown in the parentheses.

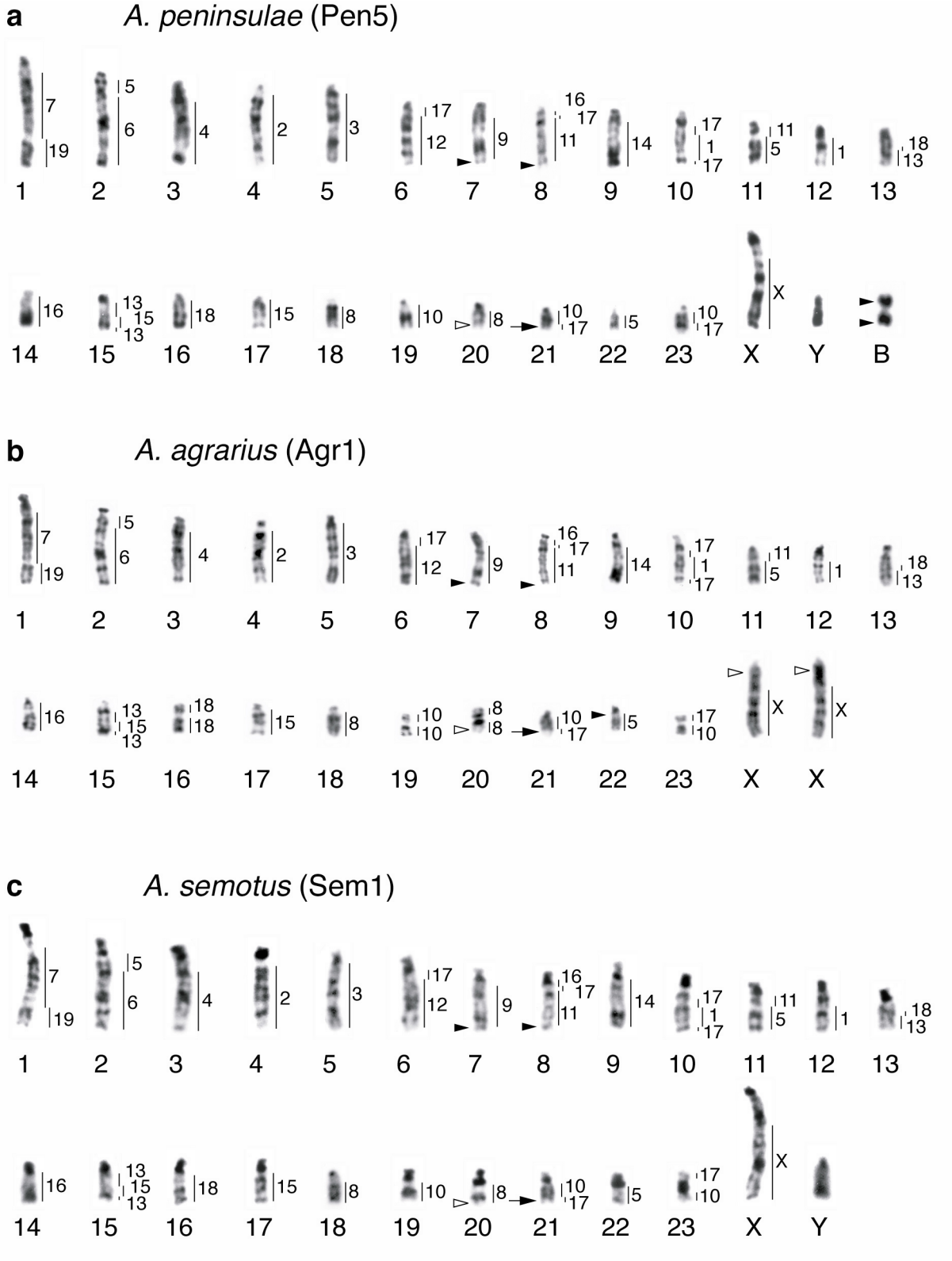

Figure 1

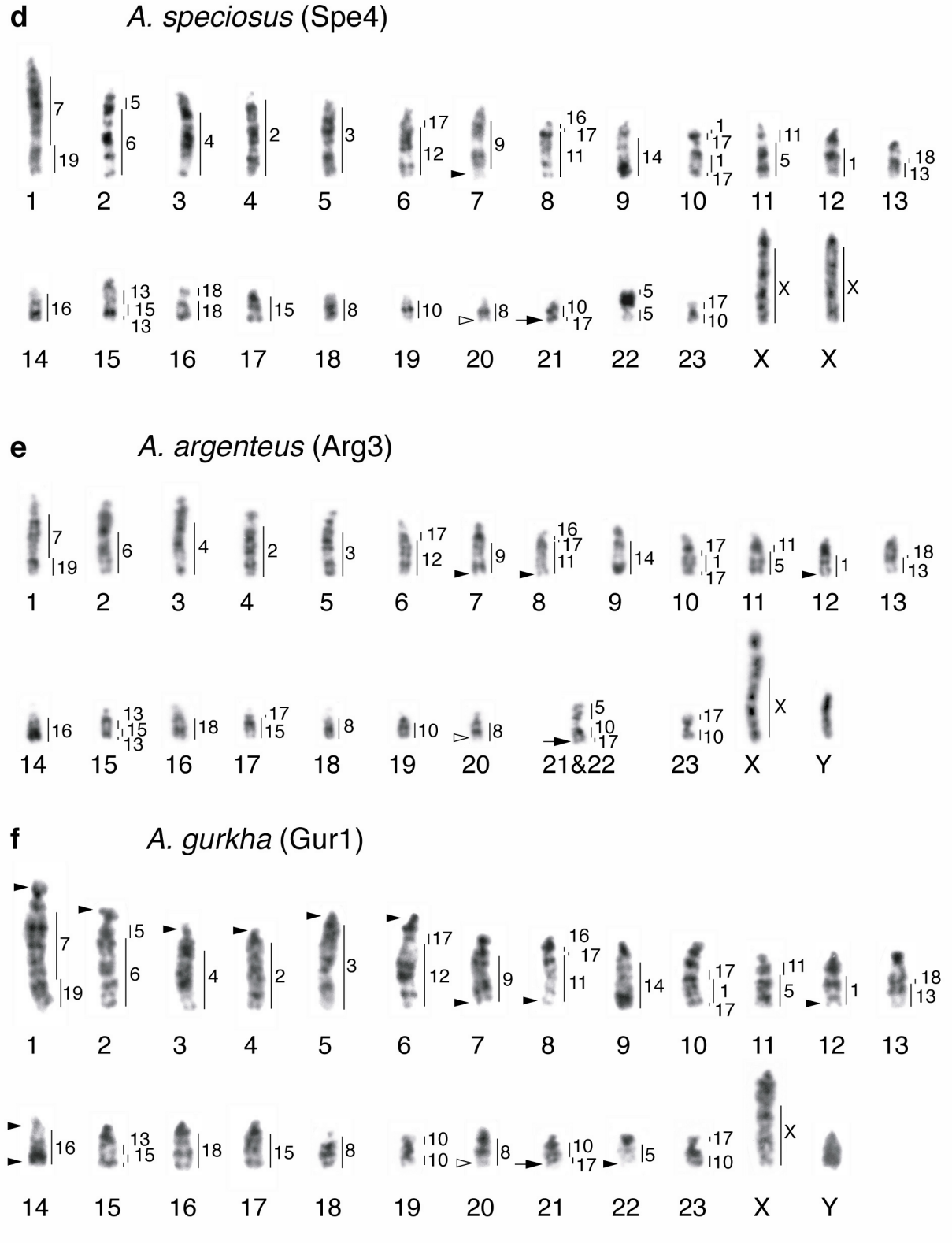

Figure 1 continued 1

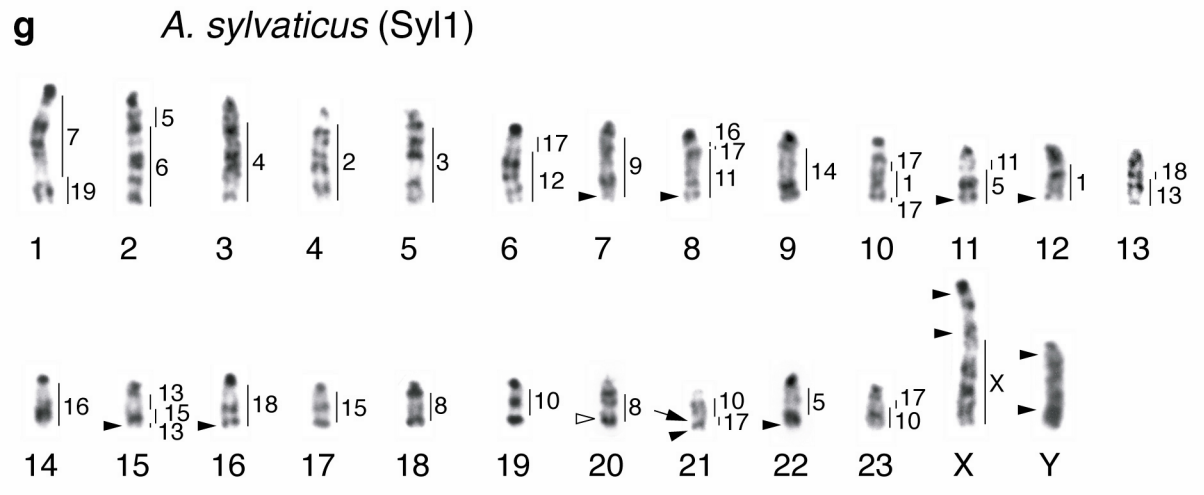

Figure 1 continued 2

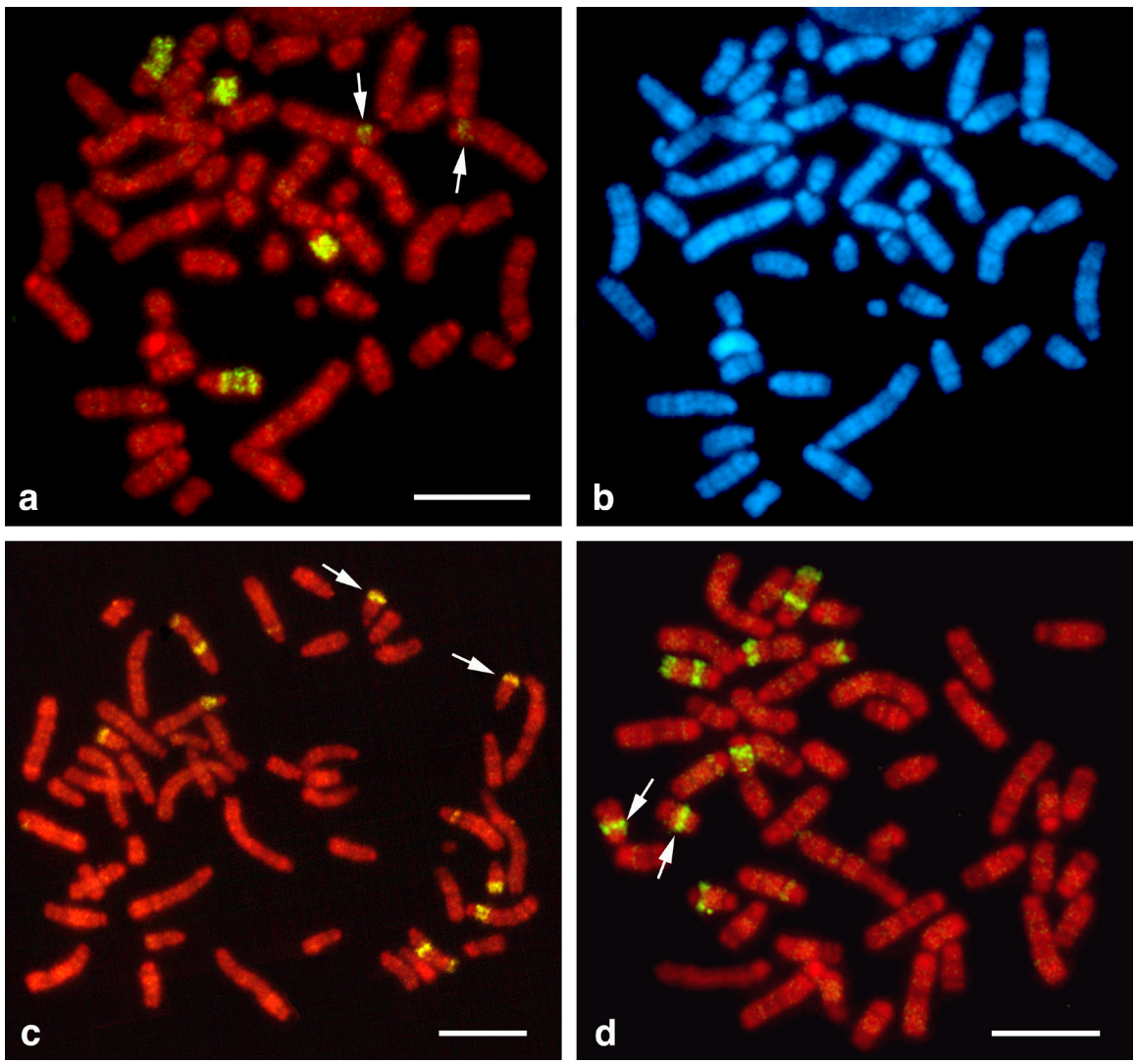

Figure 2

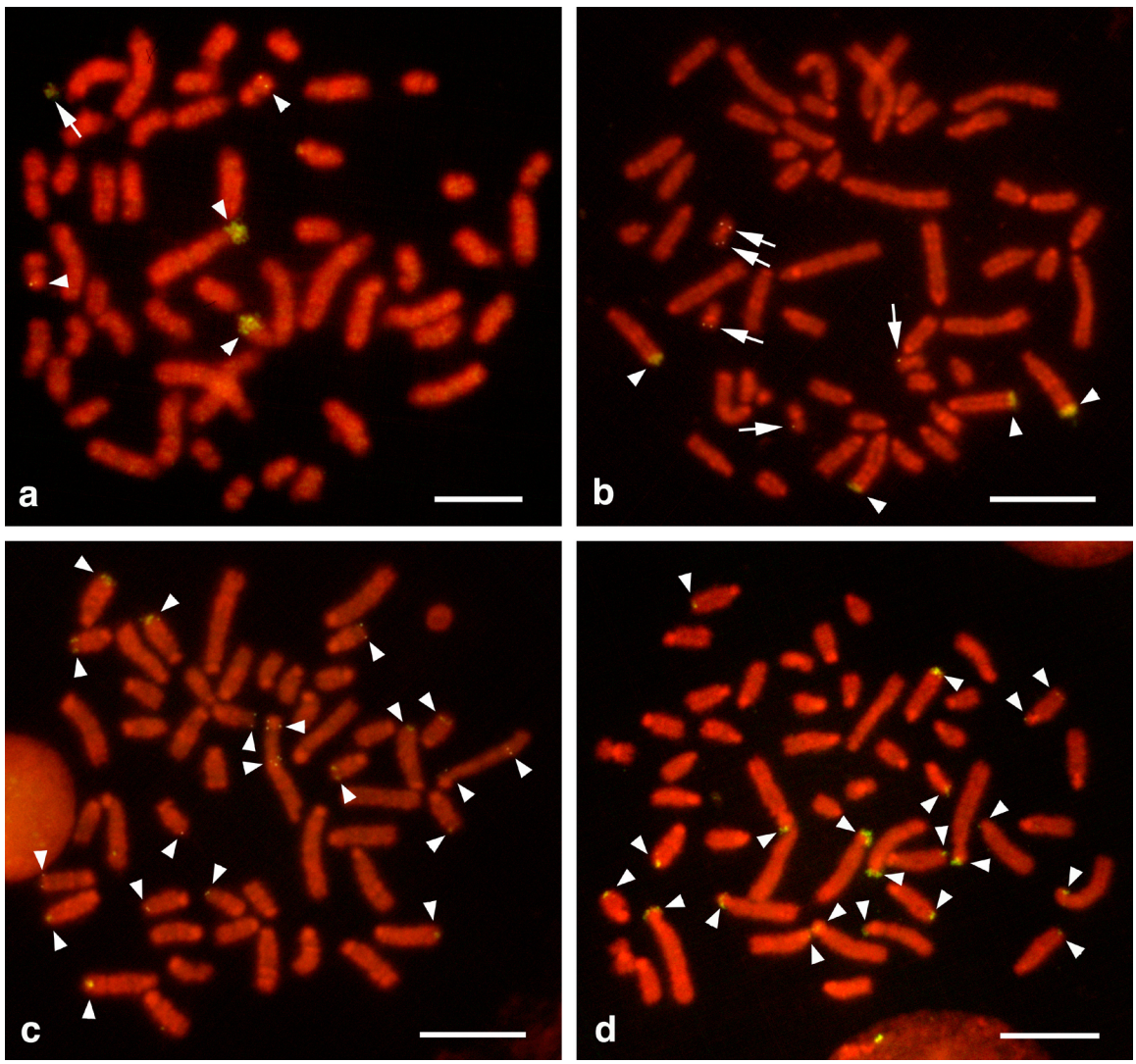

Figure 3

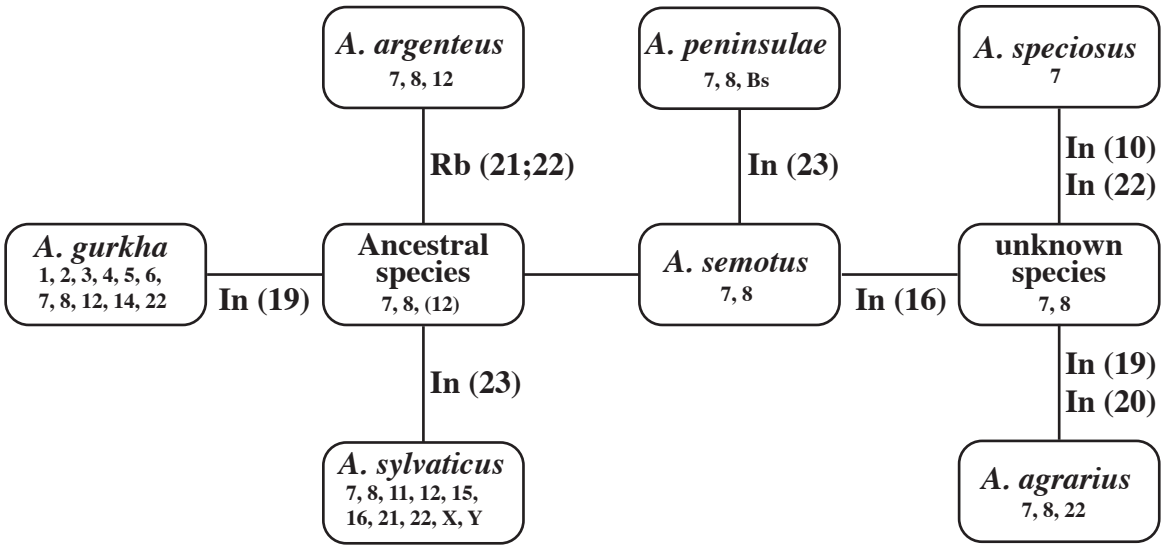
A. argenteus
7, 8, 12
A. peninsulae
7, 8, Bs
A. speciosus
7
Rb (21;22)
In (23)
In (10)
In (22)
A. gurkha
1, 2, 3, 4, 5, 6,
7, 8, 12, 14, 22
In (19)
Ancestral species
7, 8, (12)
A. semotus
7, 8
In (16)
unknown species
7, 8
In (23)
In (19)
In (20)
A. sylvaticus
7, 8, 11, 12, 15,
16, 21, 22, X, Y
A. agrarius
7, 8, 22

Figure 4