Zoology 116 (2013) 307–315

Contents lists available at ScienceDirect

Zoology

journal homepage: www.elsevier.com/locate/zool

Genomic analyses of the Formosan harvest mouse (*Micromys minutus*) and comparisons to the brown Norway rat (*Rattus norvegicus*) and the house mouse (*Mus musculus*)

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ARTICLE INFO

Article history:
Received 17 October 2012
Received in revised form 13 June 2013
Accepted 13 July 2013
Available online 28 August 2013

Keywords:
Comparative genetics
FISH
Genomic analysis
Harvest mouse
18S rRNA

ABSTRACT

The harvest mouse, *Micromys minutus* (MMIN), has a very wide range of distribution (from the British Isles across the Euroasian continent to Japan and Taiwan). We studied an isolated population of MMIN in Taiwan, which is at the southeastern margin of the species' geographic distribution, and compared its genetic complement with those of the same species previously reported from other geographic locations and with two model rodent species, the house mouse (*Mus musculus*) and the brown Norway rat (*Rattus norvegicus*). The diploid number (2N) of MMIN was 68, consistent with that reported for other populations. However, variations were noted in the fundamental number (FN) and the shape and banding patterns of the individual chromosomes among populations. The FN of MMIN was estimated to be 72, including 2 bi-armed autosomes, 31 one-armed autosomes, and one pair of one-armed sex chromosomes. Here, we propose the first ideogram for MMIN. C-banding, Ag-NOR, and the locations of 18S rRNA gene sequences (MMIN chromosomes no. 10, 14, 19, 29, 31, 33, and X) mapped by fluorescence in situ hybridization (FISH) are also reported. Additionally, we compared the 18S rDNA sequences and performed cross-species X chromosome painting (FISH) for *M. minutus*, *M. musculus*, and *R. norvegicus*. The results indicate that both genetic elements are rather conserved across species. Thus, implications for the phylogenetic position of *Micromys* were limited.

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1. Introduction

The harvest mouse, *Micromys minutus* (MMIN), has been the sole species of the genus *Micromys* until recently when a distinct species *Micromys erythrotis* was proposed for the subpopulations in Northern Vietnam and Sichuan Province, China (Abramov et al., 2009). It is among the smallest species of known mammals, with a size approximately half of the house mouse (Jiang and Lin, 2009). An adult harvest mouse weighs only ∼5–6 g and is ∼5.3–6.3 cm long from nose to base of tail. The ears are small and covered with short, sparse and pale brown hairs, and the tail is markedly discolored (brownish above and pale yellowish or mottled white below). There are many specializations, for example, a prehensile tail and rather broad feet that are adapted specifically for climbing, and a unique nesting behavior is also noted in this rodent species. In Taiwan, the harvest mouse is often found in habitats at early succession stages, e.g., establishment of grass after fire or cultivation (Jiang and Lin, 2009).

The geographical range of the harvest mouse is relatively wide, extending from northwest Spain through central Europe to Siberia, Tibet, Assam, Taiwan, and Japan (Trout, 1978). Chromosomal analyses of some European and Asian harvest mouse populations have been reported, with varying results (Makino, 1944; Tsuchiya, 1979; Jüdes, 1981; Zima, 1983; Schmid et al., 1984; Özkan et al., 2003; Nakamura et al., 2007). However, in such a widely distributed species independent chromosomal evolution among populations is plausible, due to long distances and geographic isolations.

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http://dx.doi.org/10.1016/j.zool.2013.07.001

Furthermore, chromosomal polymorphisms are not uncommon in mammals, particularly within Rodentia (Cavagna et al., 2002; Wang et al., 2003).

The island of Taiwan is located on the southeastern margin of the geographic distribution range of the harvest mouse; specimens collected in Taiwan in 1940 were used in the first karyological characterization of this species (Makino, 1944). In that report, the Formosan harvest mouse ("Formosa" is the ancient name of Taiwan from when the sailors from Portugal first saw the island) was named *M. minutus takasagoensis* and was considered to be distinct from the Japanese *Micromys japonicus*. However, only the diploid number and shapes of the chromosomes were mentioned therein.

The phylogenetic relationships of *Micromys* to other murine genera remain a subject of debate. Although DNA sequence data from a variety of genes are available, so far there has been no resolution regarding this matter (Martin et al., 2000; DeBry and Sagel, 2001; Michaux et al., 2002; Lecompte et al., 2008). During the cytogenetic assessment of the chromosomal complements, we noted that the G-banding pattern of the X chromosome of *Micromys* seems much more similar to that of *Mus musculus* than to that of *Rattus norvegicus* (see supplementary Fig. S1 in Appendix A). We thus assumed that comparing the X chromosomes of *M. minutus*, *M. musculus* and *R. norvegicus* might allow insights into the phylogenetic position of *Micromys*. Cross-species chromosomal painting (termed Zoo-FISH) provides a powerful approach for delineating true regions of chromosomal homology across species and has been extensively used for studies of karyotypic evolution in mammals (Ferguson-Smith et al., 1998; Guilly et al., 1999; Ferguson-Smith and Trifonov, 2007; Yu et al., 2012).

In the present study, we characterize the chromosomal complement of the Formosan MMIN with G-banding, C-banding and Ag-NOR staining and compare our results with those from other populations in different geographic locations. Likewise, since ribosomal DNA plays an important role in karyotypic evolution (Rakotoarisoa et al., 2000), and because the length of 18S ribosomal RNA coding sequence (rDNA) is suitable for generating the probes used in fluorescent in situ hybridization (FISH), the 18S rDNA sequence of MMIN was cloned and mapped to metaphase chromosomes in order to identify its chromosomal distribution. This FISH mapping also helps to delineate the satellites of the acrocentric chromosomes in this species. Moreover, we compared the 18S rDNA sequences and performed cross-species X chromosome painting (FISH) between MMIN and two model rodents (*M. musculus* and *R. norvegicus*) to determine whether these two genetic markers may provide insights into the phylogenetic relationships of *Micromys* with respect to *M. musculus* and *R. norvegicus*.

2. Materials and methods

2.1. Cell culture, metaphase preparation, G-banding, C-banding, and silver-NOR staining

Fibroblast cell lines were established from lung tissues derived from Formosan harvest mice (2 males and 2 females). Staining of metaphase chromosomes was performed according to published protocols (Chiang et al., 2004). For G-banding (Seabright, 1971), slides aged at 95 °C for 45 min were treated with 0.05% trypsin-EDTA for ~45–55 s at room temperature and stained with Wright's dye for 90 s. Additionally, to clarify whether satellites or tiny short arms exist in suspected acro-/subtelocentric chromosomes, a simple routine Giemsa staining was also performed. The C-banding was carried out as described by Sumner (1972), with some modifications. Slides were aged at 50 °C for 1 h, treated with 0.2 N HCl at room temperature for 1 h, rinsed with ddH_2O, treated in an alkali solution (containing 5% $Ba(OH)_2$) at 50 °C for 15 s, washed with copious amounts of ddH_2O, and finally incubated in 2× SSC at 65 °C for 1.5 h. Treated slides were stained with Wright's dye for 2 min. For silver-NOR staining (Goodpasture and Bloom, 1975), slides were treated with 2 volumes of 2% gelatin/1% formic acid and 4 volumes of 50% silver nitrate solution at 70 °C for 1–2 min. Subsequently, the slides were washed with ddH_2O, air dried, and then stained with Wright's dye for 2 min.

2.2. Isolation of 18S rDNA

Genomic DNA was extracted from whole blood of MMIN using a standard phenol–chloroform procedure (Sambrook and Russell, 2001). A primer pair MMIN18S_F: 5′-ACCTGGTTGATCCTGCCA-3′ and MMIN18S_R: 5′-TTAATGATCCTTCCGCAGGT-3′ were designed consulting the reported 18S rDNA of *M. musculus* (NR_003278) and *R. norvegicus* (M11188), and used for amplification of the MMIN 18S rDNA (1869 bp) from the genomic DNA by polymerase chain reaction (PCR). The thermal profile was set as 5 min of initial denaturation at 94 °C, followed by 30 cycles of 94 °C denaturation for 45 s, 60 °C annealing for 45 s, and 72 °C extension for 2 min, and a final 10 min extension at 72 °C. Each 25 μl reaction volume contained 100 ng of genomic DNA, 10 mM Tris–HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.1 μM of each dNTP, 0.2 μM of each primer, and 0.5 units of FastStart Taq DNA polymerase (Roche, Mannheim, Germany). The PCR products were separated on a 1% agarose gel and purified using a Gene-Spin 1-4-3 DNA extraction kit (Protech Technology, Taipei, Taiwan). The purified fragment was then cloned into the yT&A cloning vector (Epicentre Biotechnologies, Madison, WI, USA) following the manufacturer's instructions.

2.3. Sequence analysis

The 18S rDNA sequence of MMIN was aligned and compared with that of *M. musculus* (NR_003278) and *R. norvegicus* (M11188), obtained from the National Center for Biotechnology Information (http://www.ncbi.nih.gob/BLAST), using the software DAMBE (Xia, 2013). The similarity of any two sequences was estimated as: $[1-(\text{pair-wise } p \text{ distance})] \times 100\%$.

2.4. 18S rDNA FISH

The purified PCR product of MMIN 18S rDNA was labeled with Dig-11-dUTP using the Nick Translation Kit (Roche, Mannheim, Germany) and used as a probe. The FISH experiments were performed with the same slides after washing off the G-banding, with a similar protocol as previously reported (Wu et al., 2007). The G-banded slides were de-stained as follows: xylene for 5 min (three times), 100% methanol for 10 min, followed by two washes (5 min each) in 100% ethanol. The de-stained slides with chromosome metaphase spreads were denatured in 70% deionized formamide/2× SSC for 2 min at 70 °C, dehydrated in a 70%, 90% and 100% ethanol series for 2 min each and air dried. The Dig-11-dUTP-labeled probes (100 ng) were denatured for 5 min at 80 °C and placed on the slides carrying denatured metaphase chromosomes under a $24\,mm^2 \times 50\,mm^2$ coverslip. Hybridization was carried out overnight at 37 °C in a moist chamber. The slides were then washed (twice for 5 min each) in 2× SSC containing 50% formamide, 0.1× SSC with 0.1% Tween 20, pH 7.0, and then blocked with 1% BSA in 0.1× SSC with 0.1% Tween 20, pH 7.0 at 37 °C for 30 min. After blocking, slides were washed in 0.1× SSC with 0.1% Tween 20, pH 7.0 at 37 °C for 5 min, and spectrum signals were achieved with 5 μg/ml of Anti-Digoxigenin-Rhodamine, Fab fragments from sheep (Roche, Mannheim, Germany) at 37 °C for 45 min. Chromosomes were counterstained with DAPI (4.6-diamino-2-phenylindole, 0.5 μg/ml) in commercial antifade solution.

2.5. X chromosome painting (FISH)

Slides were pretreated in 2× SSC for 30 min and 0.002% pepsin solution for 3 min at 37 °C, followed by dehydration in an ethanol series (70%, 90% and 100%). For hybridization, mouse (*M. musculus*) and rat (*R. norvegicus*) WCP X probes (from Applied Spectral Imaging, Carlsbad, CA, USA and from MetaSystems, Altlussheim, Germany) were denatured at 80 °C for 7 min; pre-annealing of repetitive DNA was carried out for 60 min at 37 °C. The denatured probe was applied to the slide with denatured and dehydrated metaphase spreads, cover-slipped, sealed, and incubated at 37 °C for 72 h. After hybridization, slides were washed in 0.5× SSC at 70 °C for 2 min, followed by 2× SSC/0.1% NP-40 for 1 min at room temperature. Chromosomes were counterstained with 0.125 μg/ml DAPI

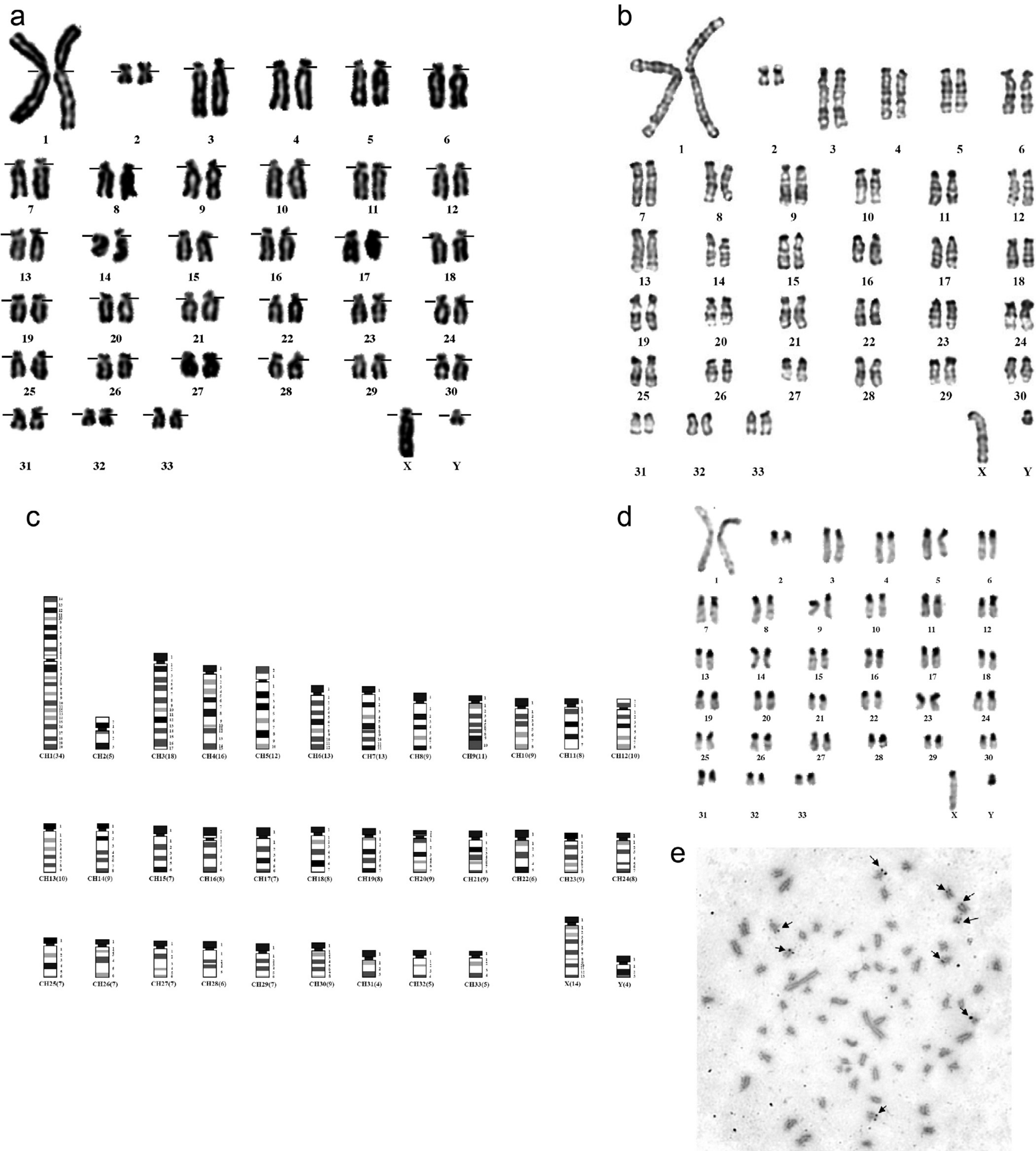

Fig. 1. Cytogenetic analyses of *Micromys minutus*. (a) Giemsa staining. – Indicates the positions of centromeres. (b) G-banding with trypsin treatment. (c) Proposed ideogram includes 331 bands (1N + XY) by G-banding. (d) C-banding. All chromosomes except pair no. 1 have prominent heterochromatin blocks in the centromeric regions. (e) Silver-NOR staining. A total of 9 nucleolar organizer regions (NORs, indicated by arrows) were identified.

added to Antifade (Vysis; Abbott Molecular, Des Plaines, IL, USA) and subsequently examined with fluorescence microscopy.

2.6. Image capture and analysis

Chromosome analyses were performed using a CytoVision Chromophour System with a CCD camera and SmartCapture software (Applied Spectral Imaging, Carlsbad, CA, USA). For FISH analyses, a fluorescence microscope, equipped with an integrated cooled CCD camera system and FISHView software (Applied Spectral Imaging, Carlsbad, CA, USA), was used.

3. Results

3.1. Diploid number and fundamental number

All specimens studied had a diploid chromosome number (2N) = 68, with fundamental number (FN) of chromosome arms = 72 and number of autosomal arms (FNa) = 70. Autosomes consisted of 1 pair of large metacentric, 1 pair of small submetacentric, and 31 pairs of acrocentric chromosomes. The X and Y chromosomes were both acrocentric (Fig. 1a).

3.2. G-banding

Multiple banding patterns were observed among metaphases in G-banding analysis (Giemsa–Trypsin–Wright method). Of those, only metaphases with banding resolutions $\geq$300 bands (1N + XY) were subjected to further karyotyping. In all chromosomes except pair no. 1, the short arms were dark-banded (Fig. 1b). Among the metaphases examined, the highest banding level was 331 bands (1N + XY). The ideogram of MMIN based on the banding level of 331 bands is shown in Fig. 1c.

3.3. C-banding

In C-banding, all chromosomes except pair no.1 had prominent heterochromatin blocks in the centromeric regions (Fig. 1d). These dark bands also extended into the whole short arms in most chromosomes. There were only minimal amounts of heterochromatin in chromosome 1. The X was the largest acrocentric chromosome in the karyotype, with constitutive heterochromatin in its pericentromeric region, as well as throughout the short arm, whereas the Y chromosome was almost completely heterochromatic.

3.4. Silver-NOR staining

There were 6–9 nucleolus organizer regions (NORs) in MMIN (Fig. 1e). Based on silver-NOR staining, the most active rDNA sites were located pericentromerically on acrocentric chromosomes. However, with the silver-NOR stain, it was not possible to definitively determine the identity of chromosomes with active 18S rDNA sites.

3.5. 18S rDNA FISH

The 18S rDNA sequence of MMIN had high sequence similarity with the 18S rDNA sequences of *M. musculus* and *R. norvegicus* (MMIN vs. *M. musculus*: 99.63% and MMIN vs. *R. norvegicus*: 99.57%) (Fig. 2). Seventeen metaphase spreads were analyzed for 18S rDNA FISH (Fig. 3) and the cytogenetic observations are summarized in Table 1. Hybridization with the 18S rDNA probe produced inconsistent results among metaphases. Fluorescent signals were quite variable in size and brightness and more frequently (>50%; see Table 1) present in MMIN chromosomes 10, 14, 19, 29, 31, 33, and X. In MMIN chromosomes 1–8, 11, 12, 15–18, 20, 22–24, 28, and Y, 18S rDNA signals were never identified. There was only one 18S rDNA site located on the telomeric long arm region of MMIN chromosome 10. All other 18S rDNA signals were located on the pericentromeric region of the corresponding chromosomes.

3.6. X chromosome painting (FISH)

The long arm of the X chromosome of MMIN was successfully and completely painted with the rat and mouse WCP X probes from MetaSystems (Altlussheim, Germany) (Fig. 4). In an initial study

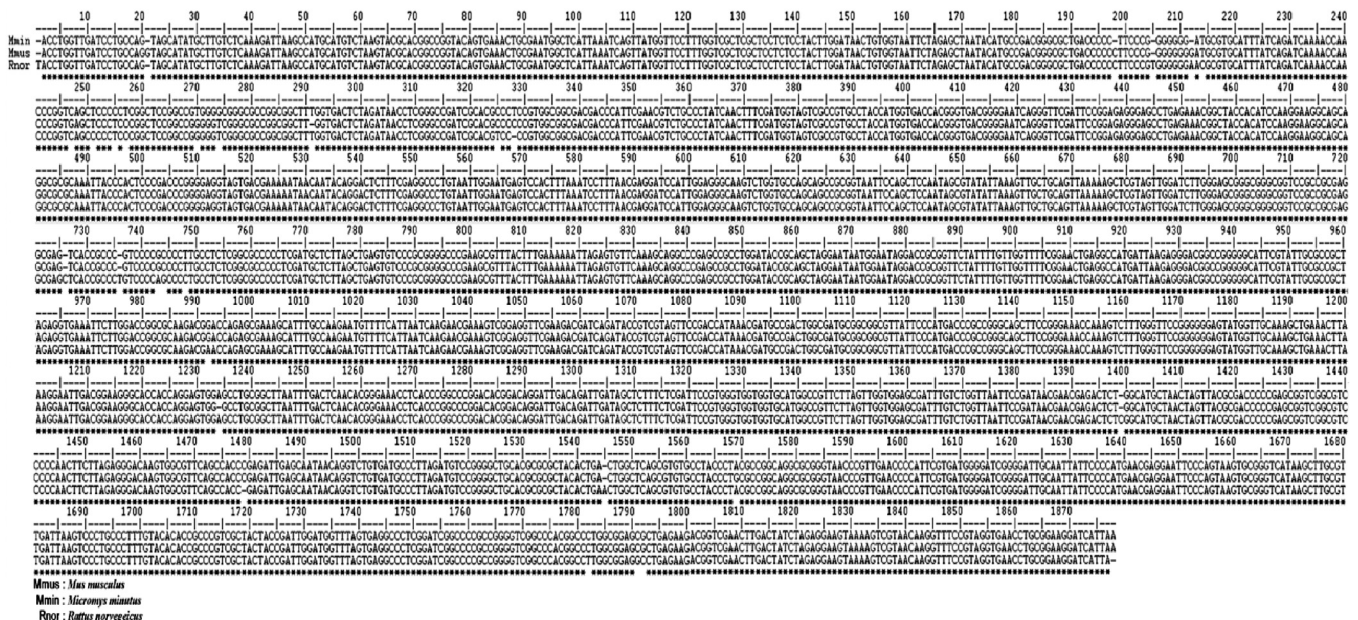

Fig. 2. Sequence comparisons of the 18S rDNA of *Micromys minutus*, *Mus musculus*, and *Rattus norvegicus*.

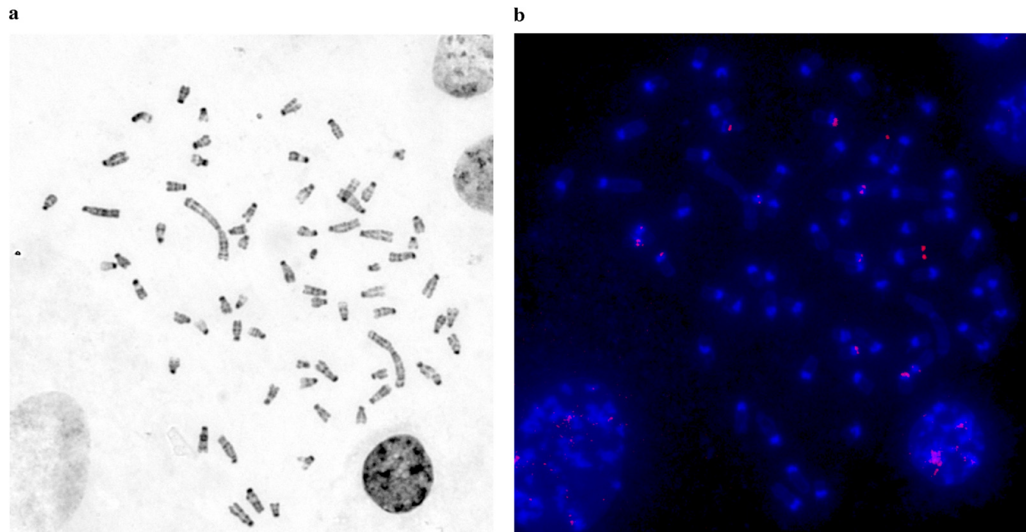

Fig. 3. Mapping 18S rDNA on *Micromys minutus* (MMIN) chromosomes. (a) G-banded metaphase spread of MMIN; (b) FISH analysis with a MMIN 18S rDNA probe on the same metaphase spread as shown in (a).

with WCP X probes from Applied Spectral Imaging (Carlsbad, CA, USA), only the rat WCP X probe had yielded results.

4. Discussion

4.1. 2N, FN, variations among the populations, and ideogram

The widespread European–Asiatic harvest mouse *M. minutus* has several cytogenetic features that distinguish it from other rodents (Schmid et al., 1987). Its high diploid number (2N = 68) was first reported by Makino (1944) and subsequently confirmed in several studies (Tsuchiya, 1979; Jüdes, 1981; Zima, 1983; Schmid et al., 1984; Özkan et al., 2003; Nakamura et al., 2007). The diploid number estimated in the present study was consistent with all of these reports. However, this diploid number is unusually high for the subfamily Murinae. In that regard, when the diploid number of this species was first reported by Makino in 1944, it was the highest among murid rodents at that time. Furthermore, the average diploid number in Murinae is 42, and only 4% of the species have a diploid number exceeding 60 (Schmid et al., 1984).

Apparent differences regarding chromosome shape are often attributed to subjective evaluation of the centromeric position in some chromosomes, as well as chromosomal polymorphism among populations. It was noted that the lengths of some short arms differed between individuals, making it difficult to accurately estimate the FN (Jüdes, 1981). In the present study, a few banding and gene mapping techniques were used to clarify the centromeric position of all chromosomes as well as the distribution of heterochromatin, and hence all chromosomes of MMIN could be classified as metacentric, submetacentric or acrocentric (no telocentric chromosomes were detected). Furthermore, no intraspecies variation in 2N or FN was noted. The controversy about FN apparently originated from the fact that different researchers used different ways of counting chromosomal arms. In our study, we decided to classify metacentrics and submetacentrics as bi-armed, and acro-/subtelocentrics and telocentrics as one-armed chromosomes to avoid confusion; thus the FN of Formosan MMIN was defined as 72. In Schmid's study, autosomes 1, 31, and Y were defined as meta- to submetacentric chromosomes, whereas all other chromosomes were classified as acrocentrics, and the authors demonstrated that the FN of this species was 72 (Schmid et al., 1984). Notably, the submetacentric autosome no. 31 of Schmid's study was similar to the bi-armed autosome no. 2 of the Formosan MMIN (Fig. 1). However, the karyotype of a Turkish population

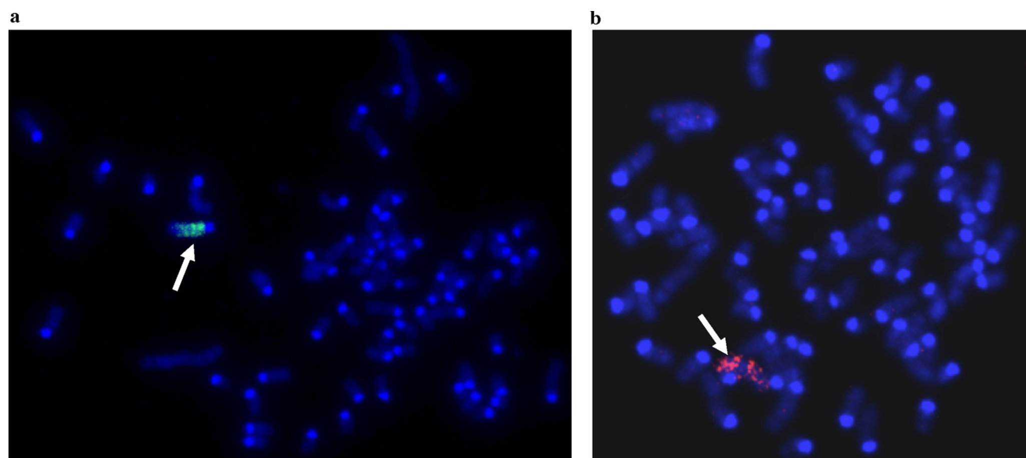

Fig. 4. Cross-species X chromosome painting of *Micromys minutus* (MMIN), *Mus musculus*, and *Rattus norvegicus*. The X chromosome of MMIN was successfully hybridized by (a) *M. musculus* WCP X probe (MetaSystems, Altlussheim, Germany) and (b) *R. norvegicus* WCP X probe. The metaphase spread is from a male MMIN and the X chromosome is indicated by an arrow.

Table 1
Summary of 18S rDNA FISH on the metaphase chromosomes of *Micromys minutus* (MMIN).

Chromosome	per ch/34	per ch (%)	per meta/17	per meta (%)	Hetero	Homo
1	0	0.0	0	0.0	0	0
2	0	0.0	0	0.0	0	0
3	0	0.0	0	0.0	0	0
4	0	0.0	0	0.0	0	0
5	0	0.0	0	0.0	0	0
6	0	0.0	0	0.0	0	0
7	0	0.0	0	0.0	0	0
8	0	0.0	0	0.0	0	0
9	4	11.8	4	23.5	4	0
10^q	22	64.7	16	**94.1**	10	6
11	0	0.0	0	0.0	0	0
12	0	0.0	0	0.0	0	0
13	1	2.9	1	5.9	1	0
14^p	12	35.3	9	**52.9**	8	2
15	0	0.0	0	0.0	0	0
16	0	0.0	0	0.0	0	0
17	0	0.0	0	0.0	0	0
18	0	0.0	0	0.0	0	0
19^p	14	41.2	12	**70.6**	10	2
20	0	0.0	0	0.0	0	0
21	1	2.9	1	5.9	1	0
22	0	0.0	0	0.0	0	0
23	0	0.0	0	0.0	0	0
24	0	0.0	0	0.0	0	0
25	5	14.7	4	23.5	3	1
26	3	8.8	3	17.6	3	0
27	5	14.7	4	23.5	3	1
28	0	0.0	0	0.0	0	0
29^p	12	35.3	10	**58.8**	6	5
30	7	20.6	4	23.5	1	3
31^p	19	55.9	12	**70.6**	7	6
32	10	29.4	7	41.2	2	3
33^p	15	44.1	11	**64.7**	9	4
X^p	15	44.1	15	**88.2**	14	0
Y	0	0.0	0	0.0	0	0

per ch/34: number of positive stainings among 34 corresponding chromosomes. per ch (%): percentage of positive stainings of the corresponding chromosome. per meta/17: number of positive stainings among 17 metaphase spreads. meta (%): percentage of positive stainings among 17 metaphase spreads; >50% indicated in bold script. Hetero: FISH signal was seen only in one chromosome of the chromosomal pair. Homo: FISH signal was seen in both chromosomes of the chromosomal pair. p: 18S rRNA genes are located on the short (p) arms of acrocentric chromosomes. q: 18S rRNA genes are located on the long (q) arm telomeric regions of chromosomes.

consisted of 1 pair of large and 3 pairs of small metacentrics and of 29 pairs of subtelo- or telocentric chromosomes. The authors claimed that the FN of this species was 136, indicating that they considered all chromosomes to be bi-armed (Özkan et al., 2003). Conversely, in Nakamura's study the chromosomes were stained with Hoechst 33258, and the authors reported that apart from the largest autosomal submetacentric pair chromosome no. 1, all chromosomes were acrocentric, and they defined the FN of the Japanese MMIN to be 70 (Nakamura et al., 2007).

One of the major reasons underlying the inconsistent results of FN seems to be the classification of the individual chromosomes as subtelo- (bi-armed) or acrocentric (one-armed). A summary of the diploid number, karyology, and fundamental number of MMIN from different reports is shown in Table 2, with the corresponding geographic locations (Fig. 5).

In the present study, homologous chromosomes of *M. minutus* (MMINC) had similar G-banding morphology. In most chromosomes, the centromere was G-positive. Furthermore, all short arms

Table 2
Karyotype comparisons among previously reported and Taiwanese populations of *Micromys minutus*.

Population	2N	FN (male/female)	Autosomes			FNa	Sex chromosomes (XY system)	Reference
			Autosome formula	Number of metacentric (m) and submetacentric (sm) chromosomes	Number of telocentric (t) and acro-/subtelocentric (a/st) chromosomes			
1. Honshu Island, Japan	68	70/70	2sm + 64a	2	64	68	X: 1a Y: 1a	Nakamura et al. (2007)
2. Kirklareli, Turkey	68	136/136	8m + 58st or t	8	58	132	X: 1t Y: 1t	Özkan et al. (2003)
3. Munich, Germany	68	73/72	4m to sm + 62a	4	62	70	X: 1a Y: 1m to sm	Schmid et al. (1984)
4. Schmilau, Germany	68	–	8m or sm + 58a/st	8	58	–	X: 1a Y: 1a	Jüdes (1981)
5. Taiwan	68	72/72	2m + 2sm + 62a	4	62	70	X: 1a Y: 1a	this study

FN, fundamental number of chromosomal arms; FNa, fundamental number of autosomal arms; –, not determined.

Fig. 5. Distribution of *Micromys minutus* (MMIN) in Eurasia (map modified from http://en.wikipedia.org/wiki/File:Distribution_of_Mycromys_minutus_Map.png). Numbers 1–5 indicate the sampling locations of the previously reported and current studies of the MMIN karyotype, as listed in Table 2.

were G-positive, except those of MMINC chromosome 1 (MMINC1), consistent with the results of a previous study (Jüdes, 1981). It is noteworthy that the current study provided metaphases with good resolution for this species. In that regard, a total of 331 bands (1N + XY) were identified and the first ideogram of MMIN was proposed (Fig. 1c), which is helpful for comparisons of the data from different populations in the future.

4.2. Repetitive DNA and karyotypic evolution

In karyotypic evolution, tandem repeats have an important role as a substrate for non-homologous recombination, thereby promoting cytogenetic rearrangements. The centromeric and subtelomeric domains appear as key regions in chromosome evolution, since both are rich in repeat sequences, harboring many breakpoints, and engaging in non-homologous recombination (Stankiewica and Lupski, 2002). The study of repetitive sequences, for example, in ribosomal DNA, is therefore of evolutionary relevance. Silver-NOR staining, C-banding, and FISH mapping were chosen to explore these repetitive sequences.

Silver only stains active rDNA on the chromosome (Miller and Therman, 2001). rRNA genes represent another family of repeat sequences, which encode the ribosomal subunits essential for translation. In eukaryotes, each unit is composed of three genes encoding 18S, 5.8S, and 28S ribosomal RNAs. In some regions, ribosomal coding elements show remarkable sequence conservation within species and even among distantly related organisms (Eickbush and Eickbush, 2007). These repetitive genes encode ribosomal RNA. The NORs may participate in nucleolus dominance, an epigenetic phenomenon in which selective silencing of the rDNA of a parent species occurs when speciation involves interspecific hybridization (Reeder, 1985). The number of NORs is influenced by transcriptional activity of rDNA and may vary due to chromosomal rearrangements. Moreover, the number of NORs may also differ among individuals, among various cell types of a single individual, or even among cells of the same slide preparation.

The NOR number of MMIN has apparently never been reported. In our study, the number of NORs in the Formosan MMIN ranged from 6 to 9. Most of the NORs were distributed pericentromerically on acrocentric chromosomes. However, since most chromosomes with NORs were of similar size, under the silver stain it was difficult to definitively determine the exact chromosome holding the NOR. Therefore, further investigations are needed to determine if there is any evolutionary relevance regarding the NOR number with respect to population differences in this widely distributed species.

In our study, by FISH mapping 18S rDNA to metaphase chromosomes to localize the satellites of the acrocentric chromosome, we uncovered that only 6 pairs of autosomes and the X chromosome stained positive for 18S rDNA. Moreover, C-banding results showed that all chromosomes but MMINC1 had prominent heterochromatin blocks in the centromeric regions. All short arms from MMINC2 were ∼33, and X was mainly constituted by heterochromatin, whereas the Y chromosome was completely heterochromatic. These findings were consistent with previous reports from Germany (Jüdes, 1981; Zima, 1983; Schmid et al., 1984). However, these characteristics reduced the value of C-banding for determining fundamental numbers. In contrast to the findings in other chromosomes, C-banding detected only a small amount of constitutive heterochromatin in MMINC1, including on the centromeric region, a phenomenon first reported by Jüdes (1981). Generally a large amount of centromeric heterochromatin is considered to be associated with a high probability of evolutionary changes. Fusions, instead of fissions, may be more likely in the evolutionary history of MMINC1 (Evans et al., 1973), and it is possible that the large MMINC1 is derived from the centric fusion of two acrocentric chromosomes from their ancestor. Similar evolutionary events were observed in other rodents such as in the genus *Rattus* (Miklos et al., 1980) and *Holochilus* (Freitas et al., 1983).

With a probe derived from the MMIN DNA, FISH identified 18S rRNA genes on chromosomes, irrespective of whether rRNA genes were active. We found that fluorescent signals were more frequently identified in MMINC10, 14, 19, 29, 31, 33, and X; for all of them, hybridized signals were detected in more than half of the chromosomes per metaphase spread. Therefore, 18S rRNA genes were more commonly located on these chromosomes. In MMINC10, the 18S rRNA gene was located on the telomeric long-arm region. In the other chromosomes, 18S rRNA genes were located on pericentromeric regions. Similarly, in other species, rRNA genes were organized into clusters present on one to several chromosome pairs and were usually located in pericentromeric or subtelomeric regions (Eichler and Sankoff, 2003). It is noteworthy

that hybridization with the 18S rDNA probe produced inconsistent results among metaphase spreads; fluorescent signals were quite variable in size and brightness. Therefore, we inferred that there was considerable variation among chromosomes in copy numbers of 18S rDNA. Specifically, for MMINC9, 13, 25–27, 30, and 32, the 18S rDNA signals were detected, but in <50% of metaphase spreads only. Perhaps the copy numbers of 18S rDNA tandem repeats in the above chromosomes were so low that the intensity of the fluorescence was not detected. Very low copy numbers of 18S rDNA have also been reported in other species (Rowe et al., 1996; Zhu et al., 2010).

4.3. Genetic markers: X chromosome and the coding sequence of 18S rRNA

Cross-species chromosome painting with chromosome-specific DNA probes is a robust method for identifying chromosome regions homologous between species at the whole-genome level (Wienberg et al., 1990). Mouse (*M. musculus*) and rat (*R. norvegicus*) are by far the most important experimental animals for human genetics and biology. Compared with other experimental animals, mice have been extensively used for genetic studies (Copeland et al., 1993), and the rat is the model of choice for human physiology, due to its relatively larger size (Jacob et al., 1995). However, because of the similar size scales of the house mouse and the harvest mouse, the mouse probe was preferentially used in the present study. An initial chromosome painting study used mouse chromosome-specific DNA probes for MMIN and reported positive findings in many small acrocentric chromosomes of MMIN (e.g., MMINC19–33), each of which was painted with a single mouse probe (Nakamura et al., 2007). It was therefore speculated that the chromosomes of MMIN (2N = 68) might be derived through fission from a presumed ancestral karyotype of the Muridae (2N = 54) during chromosomal evolution (Nakamura et al., 2007).

In the present study, both the mouse and the rat WCP X probes were successfully hybridized to the X chromosome of MMIN. This finding is consistent with the notion that the mammalian X chromosome is rather conserved when compared with other autosomes and the Y chromosome (Scherthan et al., 1994; Svartman et al., 2006; Yu et al., 2011). In previous molecular phylogeny studies using the nuclear-encoded gene IRBP, cytochrome *b*, and 12S rRNA, the position of MMIN in the phylogeny relative to other murine genera remained undetermined (Martin et al., 2000; DeBry and Sagel, 2001; Michaux et al., 2002; Lecompte et al., 2008). When comparing the 18S rRNA sequences among the three rodent species (rat, mouse, and harvest mouse), the sequences show a very high homology (>99%).

Unsurprisingly, in our study neither the mouse nor the rat Y chromosome probe yielded detectable hybridization with the Y chromosome of MMIN, a finding similar to that of Nakamura et al. (2007). It is noteworthy that the Y chromosome is highly enriched with species-specific repeat sequences and is considered much less conserved in eutherian mammals (Yu et al., 2011, 2012).

4.4. Conclusion

Our study of the Formosan harvest mouse supported the previously reported 2N number and confirmed the FN and FNa. We proposed the first ideogram of MMIN. The 18S rRNA gene of this species was cloned and mapped to the metaphase MMIN chromosomes, which may be useful for future studies of chromosomal evolution or comparisons among various populations of this species. Finally, we demonstrated that both the 18S rDNA sequences and the composition of X chromosomes are highly conserved across MMIN, *M. musculus* and *R. norvegicus*, and therefore implications for the phylogenetic position of *Micromys* were limited.

Acknowledgements

We thank Professor Hon-Tsen Yu of the Institute of Zoology and Department of Life Science, National Taiwan University, Taipei, Taiwan, Dr. En-Min You of the Department of Biotechnology, Ming Chuan University, Taoyuan, Taiwan, and two anonymous reviewers for providing constructive comments on a previous version of the manuscript. This study was supported by a grant from Changhua Christian Hospital, and a grant from National Science Council, Taiwan (NSC-99-2321-B-371-001-MY3) to Ming Chen.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.zool.2013.07.001.

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