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Reconsideration of the Karyological Relationship between Two Japanese Species of Shrew-moles, *Dymecodon pilirostris* and *Urotrichus talpoides*

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ABSTRACT—Details of the karyological relationship between the lesser Japanese shrew-mole *Dymecodon pilirostris* (2n = 34) and the greater Japanese shrew-mole *Urotrichus talpoides* (2n = 34) were examined by five differential-staining techniques, namely, G-, C-, NOR-, Q- and CMA-banding. Staining revealed that thirteen autosomal pairs and the sex chromosomes of the two species exhibited strong homology in terms of banding patterns. The remaining three autosomal pairs, nos.13, 14 and 15, exhibited distinct interspecific differences both in banding patterns and in the morphology of the respective chromosomes. These interspecific differences can be explained by the presence or absence of an unusual (G-, C-, NOR- and Q-band-negative, but CMA-band-positive) region of chromatin, by the pericentric inversion inv (14) (p13q31), and by duplication of C-heterochromatin, respectively. It has been suggested that the unusual chromatin found in *U. talpoides* might contain highly repetitive GC-rich sequences, even though its staining properties appear to be unique and are unlike those of so-called C-heterochromatin. Detailed pair-matching analyses of banded chromosomes led us to revise the relationship, proposed previously by Hamada and Yosida, between *D. pilirostris* and *U. talpoides* (Hamada and Yosida, La Kromosomo II-20, 585-590, 1980).

INTRODUCTION

The subfamily Scalopinae is among the most primitive taxa in Talpidae. It includes the lesser Japanese shrew-mole *Dymecodon pilirostris* and the greater Japanese shrew-mole *Urotrichus talpoides*, both of which are endemic to Japan. It remains unclear whether or not these shrew-moles are congeneric because of the strong similarity in external morphology and the dissimilarity in internal morphology. Imaizumi (1960) classified these two types of shrew-mole as related species that belong to distinct genera on the basis of their dental and skeletal characteristics, whereas Corbet (1978) regarded them as congeneric members of *Urotrichus*. In an effort to resolve this controversy, Hamada and Yosida (1980) compared the G- and C-banded karyotypes of specimens of these two types of shrew-mole that had been collected at the foot of Mt. Fuji, Shizuoka Prefecture. They concluded that the karyological relationship could be explained by a pericentric inversion that accompanied a large-scale deletion in the terminal half of the euchromatic long arm of a large subtelocentric pair of chromosomes, namely, no. 14. However, it is still unclear from a genetic perspective whether such large-scale deletion of chromosomal euchromatin is capable of contributing to speciation without any effect on viability since such a large-scale deletion would be likely to result in the loss of a large number of essential genes. We postulated that detailed analyses of chromosome banding by various staining techniques might enable us to resolve this issue.

Conventional karyotype analysis by Tsuchiya (1988) revealed chromosome polymorphism in *U. talpoides*, which was characterized by two different karyotypes, and he postulated that an imaginary line between Tsuruga and Owari might separate these two forms. On the basis of well-defined G-banding patterns, Ando *et al*. (1997) suggested recently that *U. talpoides* consists of two karyotypic forms (a western and an eastern form) that are separated geographically by a hypothetical line from Kurobe to Fuji in the center of Honshu and, moreover, that the karyotypic differentiation was due to a pericentric inversion in chromosome pair no. 14. They also demonstrated the karyotypic similarity between the western form and *D. pilirostris* by conventional staining. Their findings provide useful information for considerations of the karyotypic differentiation of *U. talpoides* from an ancestral form of *D. pilirostris*. To extend these findings, we made a detailed comparison of the karyotypes of *D. pilirostris* and *U. talpoides* (eastern form) using various chromosome-banding techniques that included fluorochrome banding.

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MATERIALS AND METHODS

Three females and three males of the lesser Japanese shrew-mole *Dymecodon pilirostris* (abbreviated herein as DPI) and eight females and ten males of the greater Japanese shrew-mole *Urotrichus talpoides* (abbreviated herein as UTA) were collected alive with Sherman live-traps or pitfall traps on the slopes and at the base of Mt. Iwaki and in and around Hirosaki, Aomori Prefecture. The dental formula proposed by Imaizumi (1960) was used for species identification.

Chromosome preparations were made from cultured tail or lung fibroblasts by the conventional air-drying method. For G- and C-banding, we used the ASG method of Sumner *et al.* (1971) and the BSG method of Sumner (1972), respectively. Nucleolus organizer regions (NORs) were selectively stained by the one-step method of Howell and Black (1980). Fluorochrome banding with AT-specific quinacrine mustard (QM) and GC-specific chromomycin A_3 (CMA) was performed as described by Caspersson *et al.* (1971) and Amemiya and Gold (1987), respectively. When necessary, chromosomes were stained sequentially with these two fluorochromes. For sequential staining, photographs were taken of QM-stained metaphases and then preparations were destained in running tap water and restained with CMA. Chromosomes were identified as proposed by Levan *et al.* (1964). Karyotypes were defined on the basis of a slightly modified version of the numbering system of Hamada and Yosida (1980).

RESULTS

Karyotypes of *D. pilirostris* and *U. talpoides*

The cytologic findings and analytic data for the two species of shrew-moles examined are summarized in Table 1. With the exception of heteromorphism in pair no. 13 of *U. talpoides* (UTA13), the two species exhibited no distinct intraspecific variations in chromosome banding patterns or in chromosome morphology.

The number of diploid chromosomes (2n) was 34 in both species, while the fundamental number (FNa) was 62 in DPI and 64 in UTA (Table 1). These results were essentially in accord with those in previous reports (Tsuchiya and Yosida, 1971; Tsuchiya, 1979, 1988; Hamada and Yosida, 1980; Ando *et al.*, 1987). The conventional karyotypes and G- and C-banded karyotypes of male specimens are presented in Figs. 1 and 2. We were able to identify all homologues, including

Table 1. Number of specimens, number of cells subjected to karyotype analysis and cytologic findings

Species	Number of specimens examined	Number of cells subjected to karyotype analysis						FNa	2n
		Conv	G	C	NOR	QM	CMA		
Talpidae									
Scalopinae									
Dymecodon pilirostris	3♂ 3♀	6	6	7	7	13	10	62	34
Urotrichus talpoides	10♂ 8♀	12	23	9	17	31	34	64	34

Conv, Conventionally stained; G, G-banded; C, C-banded; NOR, NOR-banded; QM, Q-banded; CMA, CMA-banded; FNa, fundamental number of autosomes; 2n, dipoid chromosome number.

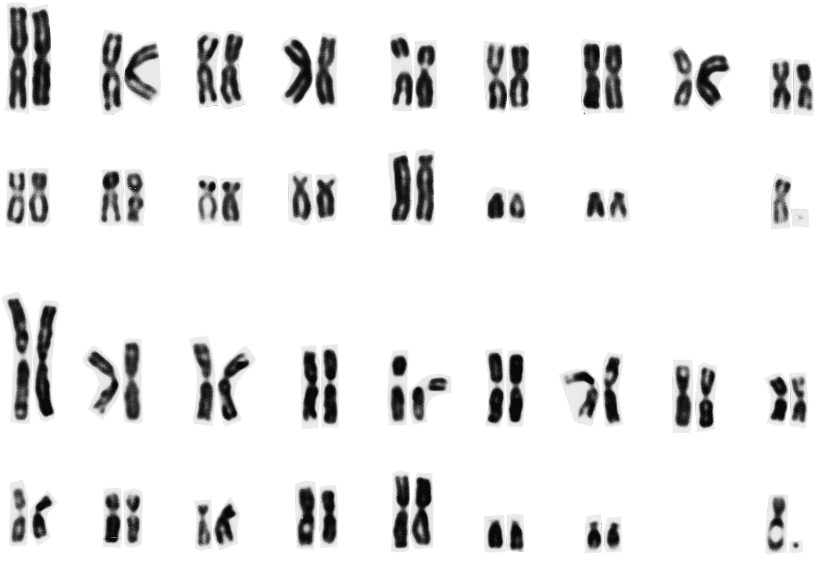

Fig. 1. Conventional karyotypes of *Dymecodon pilirostris* (a) and *Urotrichus talpoides* (b).

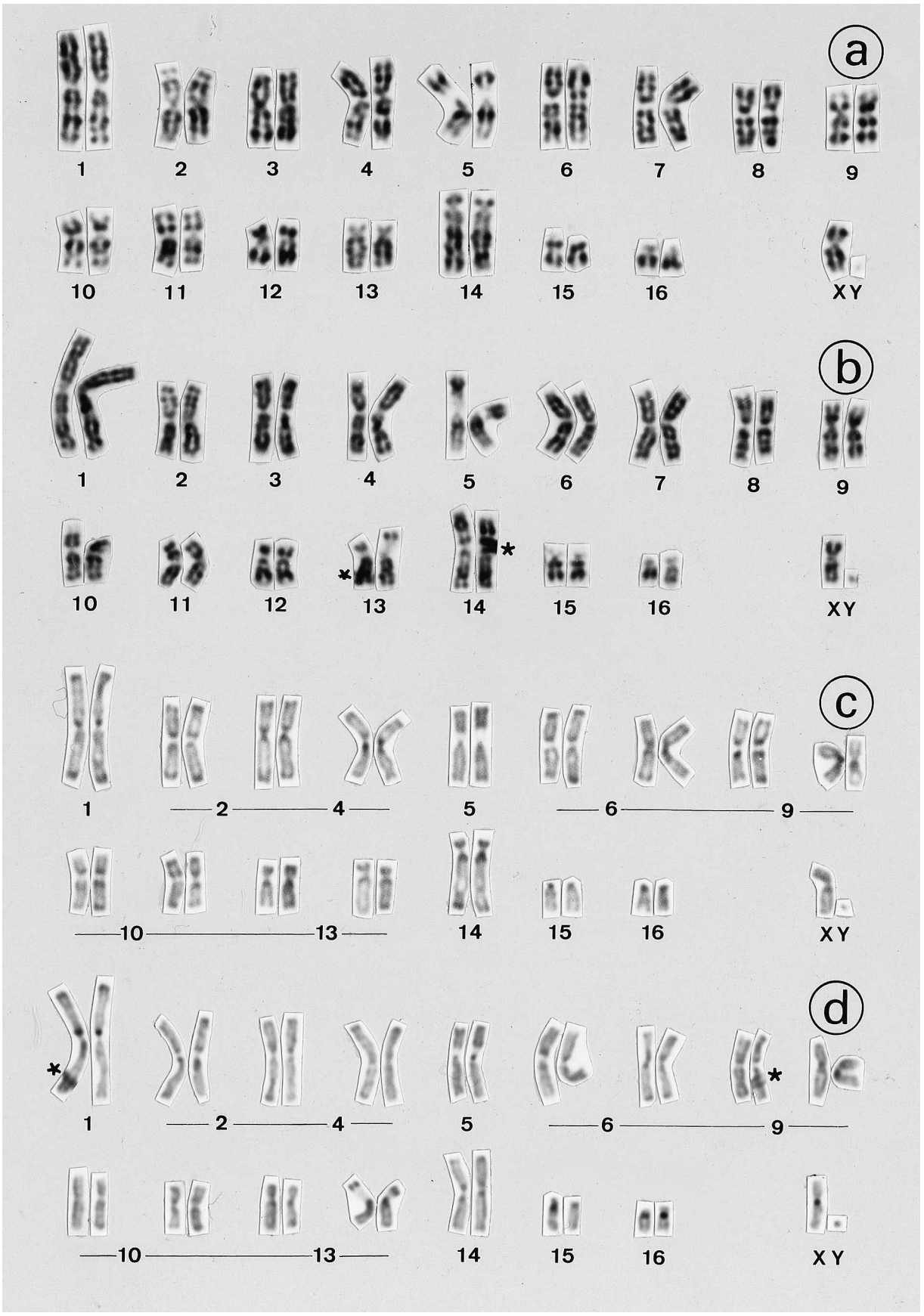

Fig. 2. G- and C-banded karyotypes of *D. pilirostris* (a and c) and *U. talpoides* (b and d). Asterisks indicate crossing of chromosomes.

sex chromosomes, on the basis of well-defined G-banding patterns (Fig. 2a and 2b). Both species have a typical secondary constriction in the proximal region of the short arm of pair no. 5 (5p), which Hamada and Yosida (1980) designated as 8p in an earlier report. The secondary constriction was unstained after both G-band and C-band staining (Fig. 2) but silver nitrate staining indicated that it was an active NOR (Fig. 3).

Interspecific differences between arm ratios (ARs) were detected in three autosomal pairs, nos. 13, 14 and 15 (Figs. 1 and 2). DPI13 was subtelocentric (AR = 3.14 ± 0.22) but UTA13 was metacentric (AR = 1.50 ± 0.11). It is noteworthy that UTA13 exhibited heteromorphism in eight of the nine specimens that we studied. One member of the pair was metacentric and the other was subtelocentric. DPI14 was subtelocentric (AR = 6.12 ± 0.72), whereas UTA14 was metacentric (AR = 1.16 ± 0.03). Intrachromosomal rearrangement(s) might explain this difference since the relative lengths of these chromosomes were almost identical (6.89 ± 0.16 vs. 6.97 ± 0.08). Unlike DPI15, which was acrocentric, UTA15 was subtelocentric.

The mean ARs of the no. 9 and X chromosomes of DPI were 1.66 ± 0.15 and 1.51 ± 0.08, respectively, and those of UTA were 1.80 ± 0.12 and 1.72 ± 0.15, respectively. These chromosomes are classified as metacentric in DPI and as submetacentric in UTA. However, the difference in ARs between species was not statistically significant and, in addition, there were no distinct differences in G-banding patterns between species (Fig. 2a and 2b). As shown in Fig. 2c and 2d, the two species yielded similar C-banding patterns although there seemed to be a slight variation in the size of C-bands between species: in both species, two smallest pairs (nos. 15 and 16) had a centromeric C-band, while the remaining autosomes lacked a centromeric C-band but did have pericentromeric C-bands to a greater or lesser extent on both sides of each centromere. The pericentromeric C-band of DPI14q was consistently much larger than that of UTA14q. Faint telomeric C-bands were detected at the ends of each chromosome. These bands were indistinct in UTA but were observed consistently from cell to cell in DPI.

Chromosome homoeology between *D. pilirostris* and *U. talpoides*

Fig. 4 shows composite karyotypes from the haploid sets of DPI and UTA chromosomes, which were arranged on the basis of G-band, Q-band or CMA-band homology. After treatment by each of the differential staining methods, all chromosomes, with the exception of the three autosomes nos. 13, 14 and 15, gave banding patterns that exhibited strong homology between species. The “homologous” pairs were regarded as intact homoeologous chromosomes. The G-banding patterns were almost identical to the Q-banding patterns (Fig. 4a and 4b), whereas almost completely reversed patterns of fluorescence were observed when we compared Q-banded and CMA-banded chromosomes (Fig. 4b and 4c).

As noted above, the G-, Q- and CMA-banding patterns of autosomes nos. 13, 14 and 15 exhibited marked variations between species (Fig. 4). The results of the various analyses of chromosome banding for these three autosomes are summarized in Figs. 5 and 6. From the characteristic banding patterns, the interspecific relationship between pair no. 14 in DPI and UTA can be explained by a pericentric inversion, inv (14) (p13q31), without any deletion of a euchromatic segment at a visible level (Fig. 5). Thus, the inversion rearrangement in the terminal segment (q31 to q35) of subtelocentric DPI14 resulted in the production of a metacentric chromosome with a G-band-

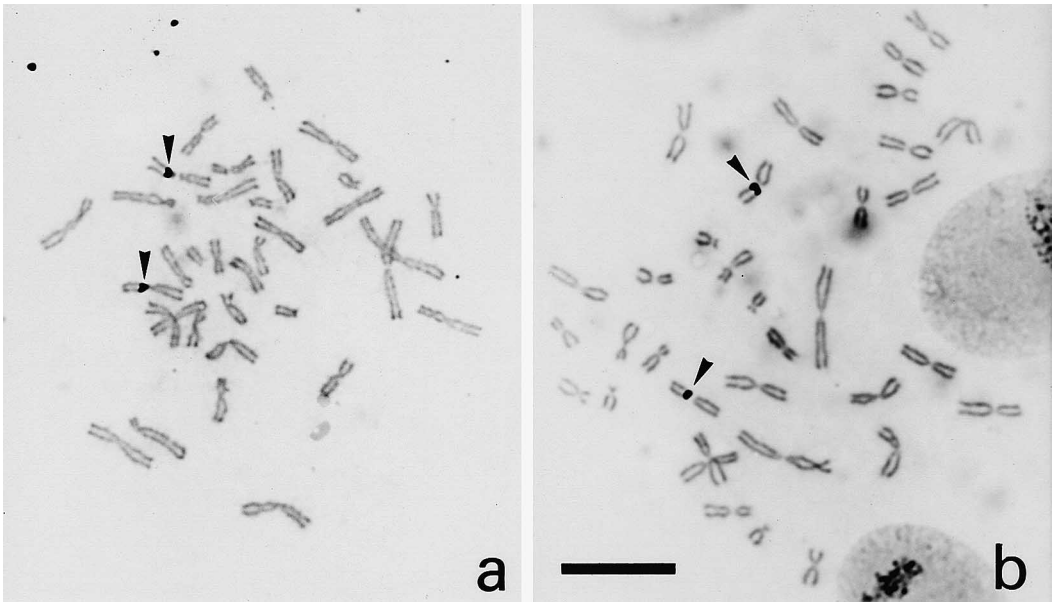

Fig. 3. NOR-banded metaphase plates of *D. pilirostris* (a) and *U. talpoides* (b). Arrowheads indicate Ag-NORs located on 5p. Bar indicates 10 μm.

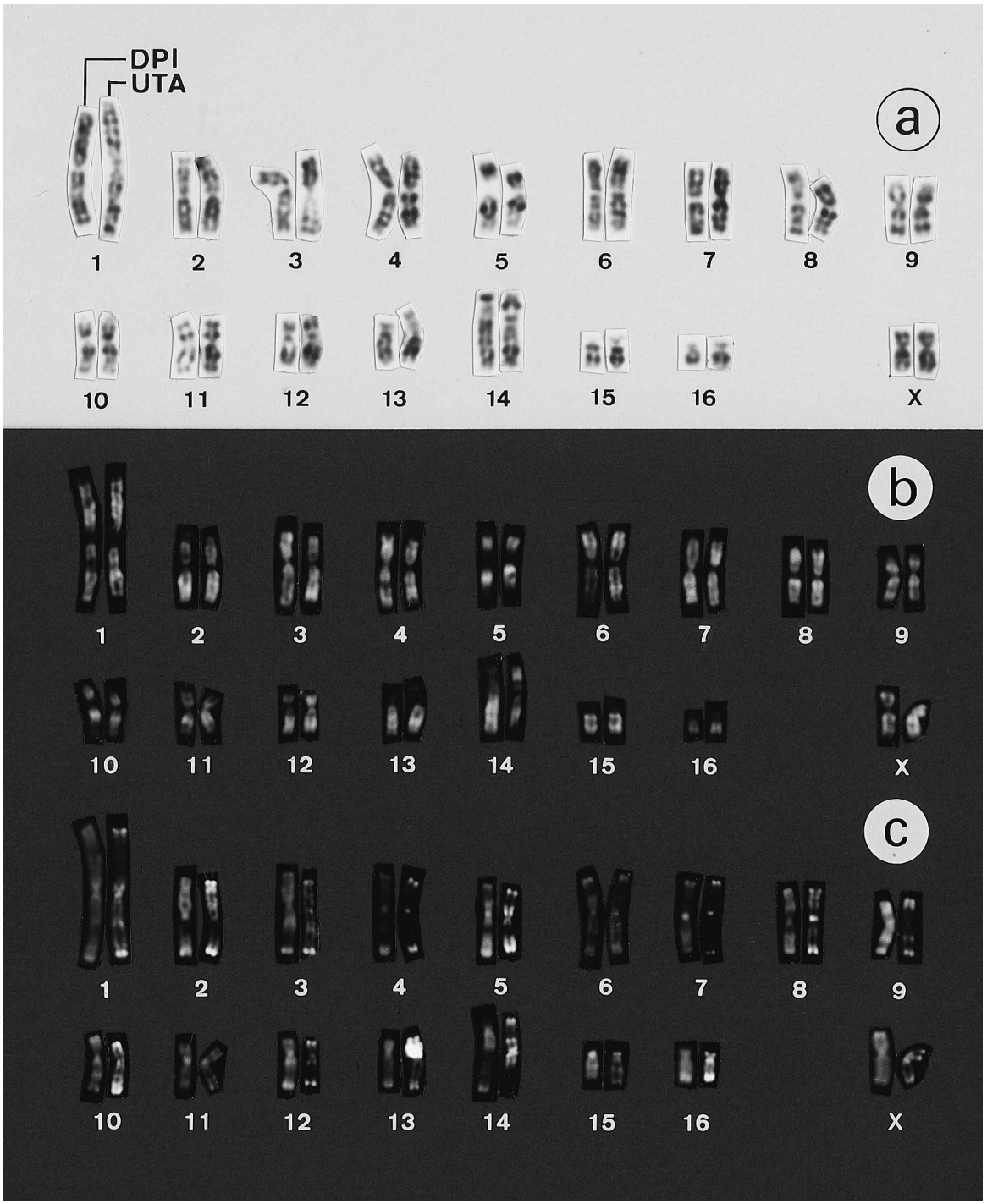

Fig. 4. Composite karyotypes prepared from *D. pilirostris* (DPI; left element in each pair) and *U. talpoides* (UTA; right element in each pair). These karyotypes were prepared by side-by-side arrangement of chromosomes with "homologous" banding patterns. a, G-banded; b, Q-banded; and c, CMA-banded chromosomes. Karyotypes b and c were prepared by sequential-staining of the same metaphase plates.

ing pattern almost identical to that of UTA14. The patterns of C-, Q- and CMA-banding provide evidence in support of this hypothesis. The finding that a spot-like CMA-band at the terminal end of DPI14 appeared to correspond to a similar band at the terminal end of UTA14p also supports this hypothesis. Similarly, the bright CMA-band at DPI14p13 might correspond to a bright proximal CMA-band in UTA14p.

Our banding analyses revealed that 13q was entirely homoeologous between species, while DPI13p was quite different in terms of both size and staining properties from UTA13p. As demonstrated in Fig. 6, UTA13 contained unusual chromatin in the proximal region of its short arm, where it emitted extremely bright fluorescence after CMA staining, but this region responded negatively to the other types of dif-

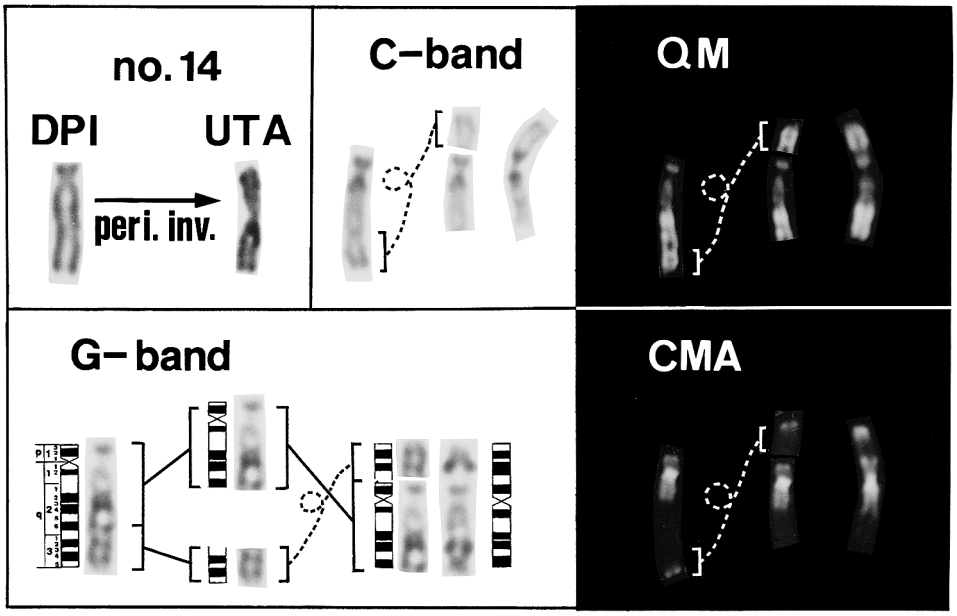

Fig. 5. Inversion rearrangement between DPI14 (left element in each panel) and UTA14 (right element in each panel). Upper row: Conventionally stained (left), C-banded (middle) and Q-banded (right) no. 14 chromosomes. Lower row: G-banded (left) and CMA-banded (right) no. 14 chromosomes.

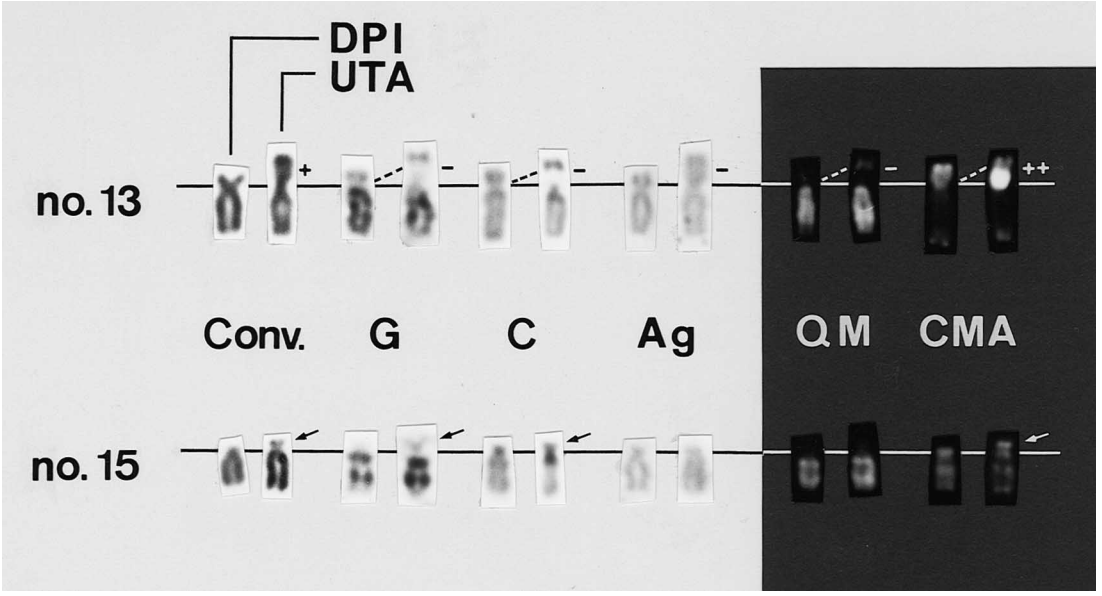

Fig. 6. Staining patterns of pairs nos. 13 and 15 after various types of staining. Conv., Conventionally stained; G, G-banded; C, C-banded; Ag, NOR-banded; QM, Q-banded; and CMA, CMA-banded chromosomes. Solid lines indicate positions of centromeres. Symbols –, + and ++ indicate no staining, normal staining and extremely bright fluorescence, respectively. Arrows indicate UTA15p. Dotted lines indicate addition of the unusual chromatin (see text for details).

ferential staining. DPI13 had no such unusual chromatin in this region at all. Chromosomes no. 15 of the two shrew-mole species was associated with a similar phenomenon: the long arms were entirely homoeologous between species, whereas the short arms differed from each other in size. The difference in size was due to the presence or absence of a small C-band that consisted of G-band, Q-band and NOR-band-negative but CMA-band-positive heterochromatin (Fig. 6).

DISCUSSION

The morphological features, such as cranial and dental characteristics, of *Dymecodon pilirostris* (DPI) and *Urotrichus talpoides* (UTA) are generally accepted as indicators that DPI is more primitive than UTA. Hamada and Yosida (1980) proposed that the only chromosomal discrepancy between DPI and UTA could be explained by a pericentric inversion that

was followed by a deletion in the terminal half of the euchromatic long arm of a large subtelocentric pair of chromosomes, no. 14. However, their study failed to confirm precise G-band homology between the two species because of the indistinct G-banding patterns that they obtained.

In this study, we found that UTA14 had sustained only a pericentric inversion, inv (14) (p13q31), without any deletion of a euchromatic segment (Fig. 5). However, this inversion might not have been the primary event that promoted speciation in the common ancestor of the two species of shrew-mole since UTA exists as two karyotypically distinct forms, western and eastern. Moreover, the karyotype of the western form is almost identical to that of DPI, having a subtelocentric pair no. 14 that seems, from the results of conventional staining, to be homoeologous to DPI14 (Ando *et al.*, 1997). Therefore, it is very likely that the inversion might have caused UTA to differentiate to two karyotypic forms after speciation.

Our detailed banding analyses also revealed that 13p in DPI and UTA (eastern form) has both different staining properties and different arm lengths, while 15p differs only in terms of arm length in the two species (Figs. 5 and 6). In pair no. 15, which is acrocentric in DPI and subtelocentric in UTA, the interspecific discrepancy in the length of the short arm might be explained by duplication of C-heterochromatin, as noted in a number of animal and plant species (Mandahl, 1978; Mandahl and Fredga, 1980; Yoshida *et al.*, 1983; Obara, 1985a,b, 1987, 1991; Siljak-Yakovlev and Cartier, 1986; Galetti *et al.*, 1991; Oshida *et al.*, 1992). By contrast, the cause of the interspecific difference in 13p seems quite unusual. As clearly demonstrated in Fig. 6, the marked interspecific difference between arm ratios of pair no. 13 (3.14 ± 0.22 in DPI and 1.50 ± 0.11 in UTA) was mainly due to the presence or absence of a region of the unusual chromatin, a phenomenon that has not been reported, to our knowledge, in any species of animal or plant. The chromatin in question, which included the proximal half of UTA13p, was unique in that it was ordinarily stained by conventional Giemsa staining, but it was negative for G-, C-, Q- and NOR-banding. Nevertheless, it fluoresced brightly after CMA staining (Figs. 4c and 6). GC-rich DNA sequences within chromosomal DNA are known to enhance the intensity of fluorescence after CMA staining (Schweizer, 1976; Schmid, 1980; Sumner, 1990). Thus, the brightly fluorescing chromatin of UTA13 (Figs. 4c and 6) might have contained a large number of highly repetitive GC-rich sequences. Such sequences might be a primary cause of heteromorphism of this region of chromatin, as is true in the case of the heteromorphic C-blocks in *Chimarrogale himalayica platycephala* and *Apodemus argenteus* (Obara *et al.*, 1996, 1997; Fukushi and Obara, 1997). The complete absence of fluorescence in this region of the chromatin after QM staining (Figs. 4b and 6) strongly supports this view. Furthermore, the absence of NORs in this chromatin (Figs. 3 and 6) suggests that it might contain GC-rich repetitive sequences other than rDNA sequences. Nevertheless, no C-heterochromatin at all was visualized after C-banding in this area. Thus, this chromatin should be regarded as "euchromatic" despite the possible presence of highly repetitive GC-rich chromatin. Alternatively, it might contain heterochromatin that is extremely sensitive to the acidic and alkaline treatments required for C-banding. An unusual type of C-heterochromatin, referred to as "C-band-negative heterochromatin", that fluoresces brightly after acridine orange reverse-banding has been detected in chromosomes of cattle, sheep and goat after staining by a modified C-banding technique (Schnedl and Czaker, 1974). Furthermore, it has been reported that, in the Eurasian common shrew *Sorex araneus*, the GC-rich chromatin, as identified by undercondensation after incorporation of the cytidine analogue 5-azacytidine (Viegas-Péquignot and Dutrillaux, 1981), is C-band-negative under ordinary conditions of C-banding, while modified milder C-banding treatment (5-15 min. denaturation in $Ba(OH)_2$ at 30°C) produces rather heavily stained bands (Schmid *et al.*, 1982).

According to Ando *et al.* (1997), UTA13 is metacentric in both the western and eastern forms of UTA and, similarly, UTA15 is subtelocentric in both forms. These facts suggest that the western form might also carry the unusual chromatin on 13p and C-heterochromatin on 15p. If this conclusion is correct, the only significant interspecific chromosomal differences are restricted to 13p and 15p. Quantitative variations in the C-heterochromatin in pair no. 15 might not have been involved in speciation of UTA, as appears to be the case in many examples reported to date, whereas the unusual chromatin of UTA13p, which was C-band- and NOR-band-negative but very GC-rich, might have had some unknown function that was involved in the reproductive isolation of populations that carried such unusual chromatin from ancestral populations of DPI. It is very likely that such unusual chromatin might be incompatible during meiosis with the short-arm chromatin of DPI13. However, the origin and functional significance of this chromatin remain a matter of conjecture. Detailed molecular cytogenetic analysis, using a DNA probe derived from this unusual chromatin, is now in progress in our laboratory in an attempt to shed some light on these issues.

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