Ann. Zool. Fennici 31:289–296 ISSN 0003-455X
Helsinki 7 October 1994 © 1994 Finnish Zoological and Botanical Publishing Board

Polymorphic and stable chromosomes of *Sorex araneus* L. differ in centromere constitution (R-banding): Evolutionary aspects

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Received 22 December 1993, accepted 22 March 1994

The karyotype of *Sorex araneus L.* (Mammalia, Insectivora, Soricidae) consists of the sex chromosome trivalent XY1Y2 and three stable metacentrics with chromosome arm combinations *cb, fa* and *ut.* The other twelve chromosome arms from *g* to *r* take part in extensive polymorphism with different arm combinations between races, different combinations of metacentrics and telocentrics between populations and individuals. The sex chromosomes, stable metacentrics and the poorly polymorphic arm pair *j/l* have moderately AT-rich centromeres. All other polymorphic chromosome arms have variable amounts of highly GC-rich centromeric heterochromatin. The GC-specific stain Chromomycin A3 (CMA) intensified with Methyl green (MG) produces a R-banding pattern with brightly fluorescent centromeric dots in polymorphic chromosomes of *Sorex araneus.* We developed a rapid *R*-band procedure with a basic buffer pretreatment (pH 8.6) followed by silver staining. This RBS-procedure first induces NOR-bands, then a succession of GC-rich cell constituents (R-bands) in the following sequence – centromeric dots – telomeric – neartelomeric – intercalary bands. This RBS-staining method is a simple tool for analysing the polymorphic chromosomes of *Sorex araneus.* The central role of the GC-rich centromeric heterochromatin in the evolution of the chromosomal polymorphism of *Sorex araneus* L. is discussed.

1. Introduction

Sorex araneus L. (Mammalia, Insectivora, Soricidae) has a karyotype with sex chromosomes XY1Y2, (*de, d, s*) three pairs of stable metacentric autosomes (*bc, af, tu*) one poorly polymorphic autosome pair (*j/l*) and a group of highly polymorphic chromosome arms *g, h, i, k, m, n, o, p, q, r* which can exist as telocentric arms or in the form of metacentrics with different arm combinations. Several tens of chromosomal races with different arm combinations are found in Eurasia

(racial polymorphism). Different combinations with metacentric (mm), telocentric (tt) or heterozygous (mt) constitution of any given two arms may be found inside a population or inside individual karyotype. Most relevant studies on the chromosomal polymorphism of *Sorex araneus* have been cited in the symposium volume on Sorex cytogenetics by the ISACC (Searle et al. 1991). In the present work we have used the nomenclature of *Sorex araneus* chromosomes suggested by the ISACC (1991), which differs from our earlier work in the exchange of the arm symbols *m* and *o* (cf. Halkka et al. 1974, Halkka et al. 1987).

We have found that the centromeric heterochromatin is moderately AT-rich in the stable chromosomes of *Sorex araneus* but highly GC-rich in the polymorphic chromosomes as shown by G- and R-banding techniques. This difference is rarely or not at all observed in Q-, G- or C-banding methods using Quinacrine, Hoechst 33258 or Giemsa (Halkka et al. 1974, Olert & Schmid 1978, Schmid et al. 1982). Volobouev & Catzeflis (1989) have used the Giemsa-based R-banding (RHG, RBG), but they did not examine the centromeric structure. Garagna et al. 1991 (with citation to Searle's thesis 1983) report on finding highly repetitive DNA in centromeric area of all other but the three oldest metacentrics of *Sorex araneus*. The DAPI or Methyl Green enhanced Chromomycin A3 technique (Schweitzer 1976, procedure 3.34.2. in Verma & Babu 1989) is specific for highly GC-rich chromatin and is an excellent method for demonstrating the difference in centromeric structure between the stable and polymorphic chromosomes of *Sorex araneus*. The procedure is time consuming, however, with long stain incubations of several hours and a long stabilization of the stained preparations of a few weeks. A fluorescence microscope is also needed.

Denton et al. (1977) have described a simple silver staining method for the detection of kinetochore structures in human chromosomes. The chromosomes were pretreated with NaOH (at pH 8.5) before silver staining. This staining resulted in the succesive appearance of NORs and kinetochores. We improvised a new modification for *Sorex araneus* chromosomes based on this method. The centromeric areas of the polymorphic chromosomes contain silver reacting material that is not found in stable chromosomes. Fully developed silver banding corresponds to the R-banding (highly GC-rich chromatin) visualized by Chromomycin A3/Methyl Green staining (CMA/MG). The silver staining procedure is described in detail below. The abbreviation RBS-banding (*R*-banding with base (pH 8.6) using silver staining) is used here following the principle of the 1971 Paris conference for chromosome banding nomenclature. Almost identical *R*-bands were also produced after acidic pretreatment and silver staining (RAS-bands).

A detailed cytochemical and molecular analysis (restriction enzyme digestions etc.) on the nature of the centromeric heterochromatin of *Sorex araneus* is being undertaken (Halkka & Vakula, in preparation). We expect that a specific class of DNA-satellites will be found corresponding to this highly GC-rich centromeric heterochromatin. This chromatin most probably plays a central role in the evolution of the extensive chromosomal polymorphism of *Sorex araneus*.

The population genetical and geographical aspects of the chromosomal polymorphism fall outside the scope of the present article. These aspects are discussed eg. in Fredga & Nawrin (1977), Searle et al. 1986, Halkka et al. 1987 and 1994, Bengtsson & Frykman (1990), in several articles of the ISACC symposium volume from 1990 (ed. Hausser, 1991) and 1993 (forthcoming), reviewed lately by Brünner (1991) and Wojcik (1993) and in the extensive literature cited therein.

2. Material and methods

Staining experiments were made on few specimens of *Sorex araneus* L. collected in 1990 from Kilpisjärvi (FIN-race N, Finnish Lapland), but the population balance of Kilpisjärvi was observed from 21 specimens collected from 1989–92 (cf. Halkka, et al.1994). The staining experiments of the Tvärminne population (FIN-race IV, Southern Finland) are based on a few specimens collected in 1992, but the data on population balance has been accumulated from a few hundreds specimens collected between 1982 and 1993. Spleen cell metaphases of *Sorex*- material were prepared as earlier

described (Halkka et al. 1987). 1–2 weeks old chromosome slides were treated individually according to the following protocols. Too fresh and too old slides behaved irregularly and seldomly produced the expected banding pattern.

Chromomycin A3/Methyl Green staining (CMA/MG)

R-banding with Chromomycin A3/Methyl Green (CMA/MG) was performed according to protocol 3.34.2. in Verma & Babu 1989. The Chromomycin A3 staining time was extended overnight and Methyl Green-staining to 1–3 hours in the dark. The embedded preparations were aged for one to several weeks at +37°C before photographing with an Olympus BH-2 fluorescence microscope.

R-banding with multistep silver staining (RBS, RAS)

R-banding with silver was performed with silver reagents of Howell and Black (1980) (see also protocol 3.22.4. in Verma & Babu 1989). The quality of the banding is dependent on the basic (or acidic) pretreatment of the slides and on the silver nitrate lot used. We have tested about 5 different $AgNO_3$-products, and the best results were obtained with a very old lot with large rosy crystals of silver nitrate (product 5023, Nitrate d´Argent, Union Chimique Belge, S.A. Bruxelles). Even one year old solutions of a good lot gave excellent staining results. Some other lots produced strong precipitate of silver over the whole preparation in all experimental conditions.

The RBS-banding with silver was optimized for *Sorex araneus* preparations as described below. All procedures apart from staining were done at room temperature; the warm plate was protected with a thin plastic sheet; gloves were worn when staining with silver.

Procedure. (1) The slide was incubated for 2–3 minutes in a 67mM Na_2HPO_4 solution (pH 8.6, basic component of the Sørensen buffer). (2) Then it was rinsed throughly in running tap water followed by two rinses in destilled water to remove any traces of the phosphate buffer. The excess water was shaken off. (3) Next it was stained with silver reagents of Howell and Black (1980). 4 drops of gelatin-formic acid solution and 4 drops of silver nitrate solution were pipetted only on the slide and it was covered with a long cover slip. A more even staining result was obtained by premixing the stain components in a clean Eppendorff tube just before pipetting on the slide. (4) Subsequently the slide was incubated on a warm plate at 40 to 60°C until a golden yellow shade of the silver stain was developed. The time was dependent on the temperature used. (5) The cover slip was rinsed off and the slide washed in running tap water followed by two rinses in destilled water. (6) Then a cover slip was put on and the staining reaction was followed under a phase contrast microscope. (7) Steps 3–6 were repeated 1 to 3 times until the required bands were developed in the chromosomes. After the last washing, the optimal staining shade of the slide was golden yellow with a faint rosy shade, and the staining of chromosomes was yellow with a dark brown shade only in GC-rich areas. (8) The slide was dried in air or in ethanol, and (9) mounted in "Entellan neu" (Merck 7961 for phase contrast preparations). (10) The slide was then analysed and photographed with an Olympus BH-2 microscope with or without phase contrast optics.

Staining experiments were done also with the acidic component of Sørensen buffer (67 mM KH2PO4, pH 4.4), and almost identical R-bands were also produced by this method (RAS-bands, see results).

Technical comments. The multistep silver staining (eg. 2+2+2 min at +60°C) gave a much better staining result than an extended one-step staining (eg. 5 min at +60°C). When the silver stain develops from dark yellow to a brownish shade, a strong silver precipitate appears in the slide background and over the metaphases. This can be avoided by multistep staining which gives clearly banded metaphases on a clean background. Throughly washing with a rich flood of tap and distilled water after buffer treatments and between the staining steps prevents unwanted silver precipitation into the background, but allows successive silver binding into specific chromatin areas.

3. Results and discussion

General aspects on G-, C-, R- or silver banding, and the few earlier cytochemical studies on *Sorex araneus* have been dealt with in the Introduction. The R-banding methods using CMA/MG and the rapid (10 minutes) RBS-staining are very useful tools in the analysis of the chromosomal polymorphism and evolution of *Sorex araneus*.

3.1. Chromosome banding reactions

Silver staining (Howell & Black 1980) of *Sorex araneus* chromosomes in the same conditions but without basic buffer pretreatment produces only NOR-bands (Table 1, column a). The NOR-reaction is found in 4–5 distal tips out of the eight NOR-carrier arms *q, o,* and *tu* of a diploid karyotype (Fig. 1a, cf. also Halkka & Söderlund 1987, ISACC 1991).

In RBS-staining, after basic buffer pretreatment, additional silver positive bands develop gradually (columns b–d, Figs. 1 b–d). First to appear are the centromeric and telomeric bands, followed by the broad neartelomeric bands and lastly the intercalary bands. Kinetochore staining (cf. Denton et al. 1977) was seldom seen and only in "ghostlike" unbanded chromosomes. The silver reaction with basic (or acidic?) pretreatment is preferential to changed DNA/protein conformation (partial denaturation) of chromatin better than to specific base sequences of DNA (Denton et al. 1977). Optimal R-banding was produced using a 2 min basic buffer (pH 8.6) treatment and golden to rosy yellow staining shade (RBS; column c). The 2 min acidic buffer (pH 4.4) treatment alone also produced banding (RAS; column e) with clear NORs and GC-rich centromeric bands, partial main R-bands, but weak telomeric bands.

It is obvious, that the fully developed bands produced by the RBS-staining (Fig. 1e) correspond to the highly GC-rich R-bands produced by the CMA/MG-staining (Fig. 1f, Fig. 2). In good preparations the bands produced by RBS-staining are even sharper and have more internal details. The intercalary R-bands help in chromosome arm identification.

Table 1. Optimizing the RBS- (and RAS-) staining treatments for studying the *Sorex araneus* chromosomes. Abbreviations: NOR = nucleolus organizer. Full R = full R-banding pattern which includes here the GC-rich components: C = centromere (only in polymorphic chromosomes), T = telomere, I = intercalary main bands. K = kinetochore.

	a	b	c	d	e
pretreatment	no	basic	basic	basic	acidic
	–	"under"	optimal	"over"	?
	–	1 min	2 min	3 min	2 min
Status of		slighty	partially	mostly	partially
chromatin	closed	open	open	open	open
and		GC/C,T	GC/R	GC,AT	GC/R (T–)
NOR	open	open	open	open	open
Ag-staining	NOR	NOR	NOR	–	NOR
pale yellow			C, T (I)	C, (I)(T–)	
	pale	pale	good		good
Ag-staining	NOR	NOR	NOR	NOR	does not
golden to		C, T(I)	full R	T, K?	develope?
brownish	good	diffuse	good	swelled	
Ag-staining	NOR	NOR	NOR	–	–
brown		C,T (I)	full R		
	dark	dark	dark		

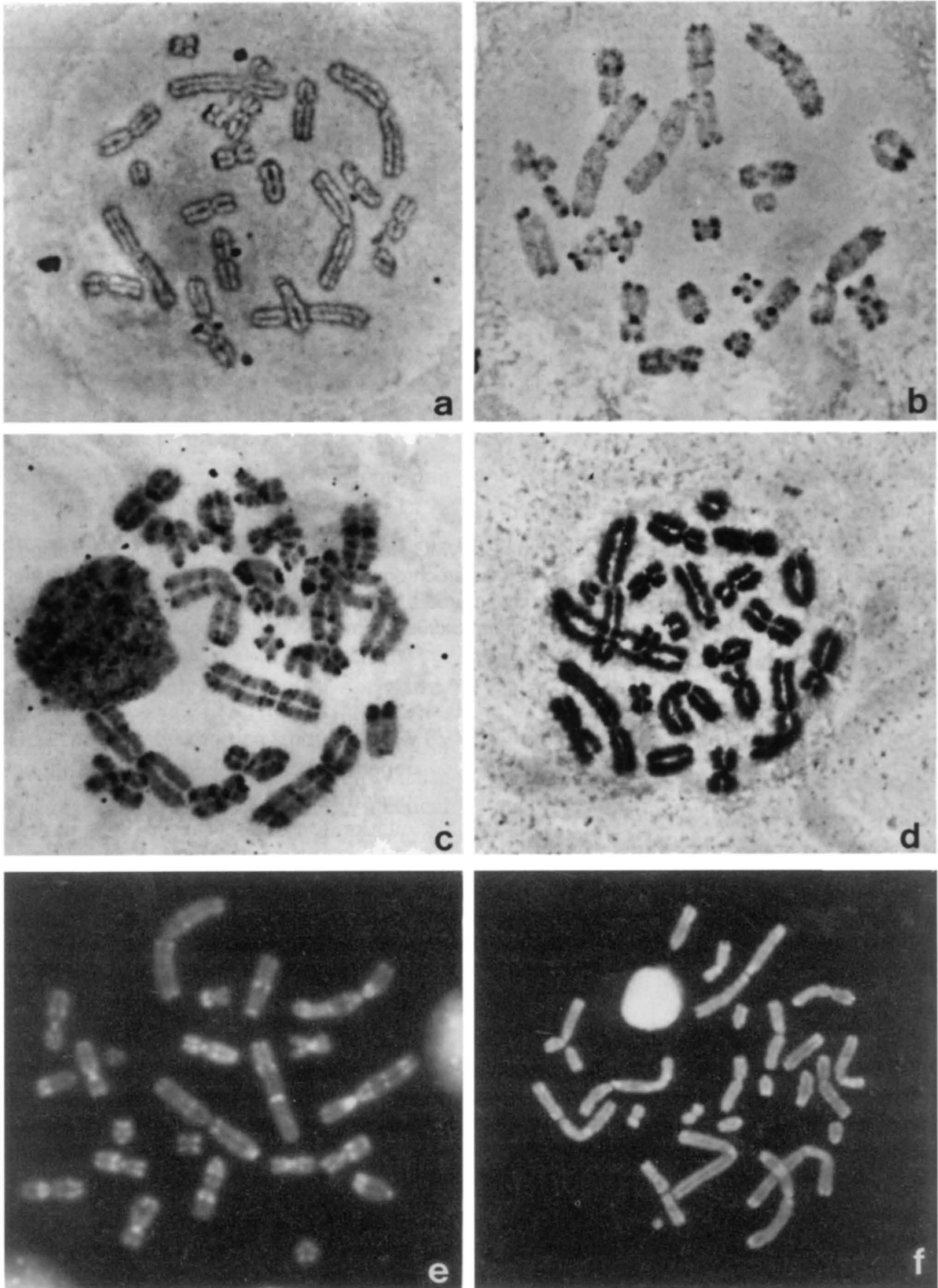

Fig. 1. a–f. Chromosome staining reactions of *Sorex araneus* specimen Sx9023 from Kilpisjärvi, race N (a–e), and Sx8570 from Lauttakylä, race IV (f). a–d: Silver staining reactions after pretreatment with basic buffer pH 8.6 for 2 min. a – Only NORs are found in pale yellow preparations. b – Centromeric and telomeric bands develop after NOR in golden yellow preparations. c – Intercalary bands developed in rosy to brownish yellow preparations. d – R-bands disappear with overstaining by silver. e – Chromomycin A3/Methyl Green staining produces fluorescent R-bands in highly GC-rich chromatin areas. f – Staining with CMA alone gives a general GC-fluorescence along the chromosomes.

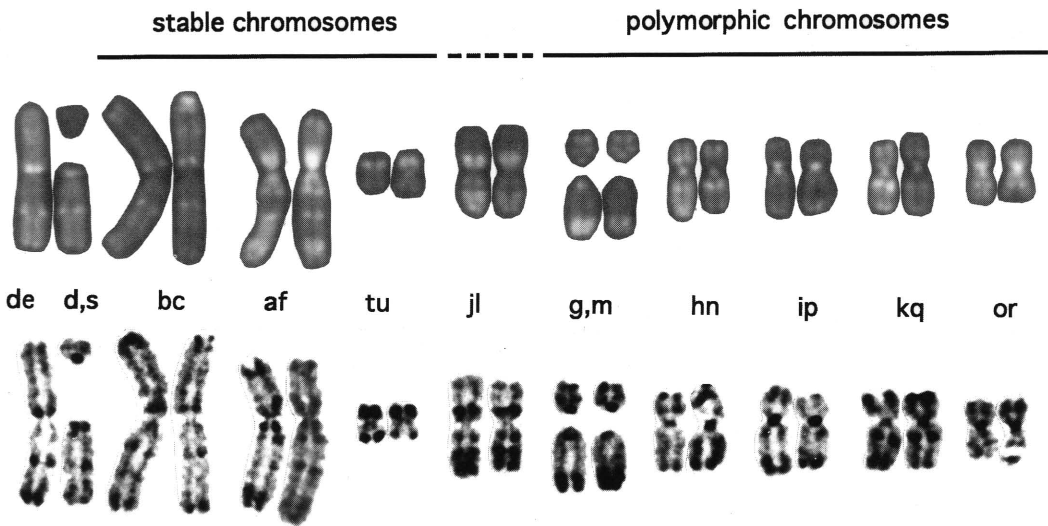

Fig. 2. Comparison of the R-bands produced by the CMA/MG-staining (upper row) and the RBS-staining (lower row). Identically located R-bands of highly GC-rich heterochromatin are produced by both staining methods. Polymorphic chromosomes have GC-rich centromeric areas, but the stable chromosomes do not. Additional NOR-spots are produced with silver staining. Specimen Sx9023 from the Kilpisjärvi population.

3.2. Centromeric reactions and heteromorphism

R-banding with CMA/MG or with silver (RBS, RAS) show two important things concerning the chromosomal polymorphism of *Sorex araneus* :

1) The stable chromosomes *bc, af, tu,* the sex chromosomes X and Y2 and the poorly polymorphic *j/l* do not have silver reacting centromeric heterochromatin. On the contrary, the centromeric areas of Y1 and the polymorphic chromosomes react intensely in RBS-banding. The polymorphic metacentrics have mostly one dot-like centromeric band, but sometimes they have a twin band.
2) *Heteromorphism* in the amount of the GC-rich centromeric heterochromatin was observed inside the same karyotype (eg. in arms *g* in specimen Sx9023, Figs. 1–2), inside a population and between races. Generally, a rich amount of highly GC-rich centromeric heterochromatin in arm pairs seems to be correlated with a high level of population polymorphism (as. in Kilpisjärvi population, Northernmost part of Finland; Fig. 2). Highly repetitive centric heterochromatin was reported also by Gagarina et al. 1991 (with citation to Searle's thesis 1983) in polymorphic populations of *Sorex araneus* in England. Low levels of polymorphism seems to be correlated with a small amount of heterochromatin in arm pairs or telocentrics (eg. in the Tvärminne population, Southwestern coast of Finland). Different chromosome arms (as extended to chromosome pools of populations and races) have thus different internal premises for chromosomal polymorphism and racial evolution. Statistical analysis on this heteromorphism has been started in the Finnish *Sorex araneus* populations.

The centromeric C-bands are difficult to reproduce with Giemsa-staining (CBG). Unevenly reacting tiny bands are found in polymorphic chromosomes of *Sorex araneus* in Finland (unpublished observations). Giemsa-based C-bands are lacking from a German population, however (Schmid et al. 1982). This may depend on the lack of a GC-rich centromeric chromatin from the non-polymorphic telocentric chromosomes

of the Ulm population in question. Zima et al. (1988) have constructed a mathematical model for all possible arm combinations found in racial evolution, based on even chance for each arm to reorganize. We think, that the chances are not even, but depend on how much and which kind of highly GC-rich hetrerochromatin is available in the centromeric area of each telocentric arm (to be fused) or metacentric chromosome (to be translocated).

The total lack of GC-rich centromeric heterochromatin from arm pair *j/l* corresponds to an almost total lack of racial arm exchanges in Eurasia. Only one exception is known from the Swiss Alps (Valais; eg. Hausser et al. 1991). This indicates that the GC-rich centromeric heterochromatin is needed to produce internal and racial polymorphism in *Sorex araneus* L.

3.3. Evolution of chromosomal polymorphism, a personal vision

The chromosomal polymorphism of *Sorex araneus* depends most probably on the highly GC-rich centromeric heterochromatin found only in the polymorphic chromosomes. Apparently these centromeres contain at least partially identical repetitive base sequences. Unequal meiotic or somatic crossing over may enrich or reduce the amount of repetitive centromeric heterochromatin and produce observable heteromorphism (reviewed eg. in Verma 1990, Lewin 1990, Voght 1990, Hamilton et al. 1992). Centric fusions, fissions and reciprocal translocations might also occur frequently inside this highly repetitive chromatin. The intercalary highly GC-rich bands (interstitial heterochromatin) found in many chromosome arms of *Sorex araneus* may represent eg. sites of ancient tandem arm fusions (residual telomeric sequences) or other structural rearrangements during the evolution of the *Sorex araneus-arcticus* group.

The stable autosomal arm pairs *af, bc, tu* lacking centromeric GC-rich heterochromatin are of old origin and are found in several species of the *araneus-arcticus* group (cf. Hausser et al. 1985, Volobouev & Catzeflis 1989, Wojcik & Searle 1988 and literature cited therein). The arm combination *jl* lacking GC-rich centromere is also very original and found in several *Sorex* species. The *j/l* arm combination is only slightly polymorphic in the species *Sorex araneus*, and found in this arm combination throughout Eurasia. Only one exceptional combination *jh – ol* has been found from Valais, Switzerland (Hausser et al. 1991, and earlier works cited therein).

Apparently the primary insertion of GC-rich sequences into centromeric chromatin caused an evolutionary burst leading to the evolution of the polymorphic species *Sorex araneus*. DNA-satellites of transposon origin are known to be connected eg. with chromosomal polymorphism and rapid evolution of *Peromyscus maniculatus* (Wichman et al. 1985) and many other organisms.

The GC-rich heterochromatin spread and accumulated first in centromeric areas of the telocentric arms. A series of centromeric fusions led to formation of new metacentrics characteristic to Western and Eastern phylogenetic groups and several more areal or local arm combinations. Maintenance of the internal polymorphism (one metacentric/two telocentrics) might be based on the succesful adoption of the meiotic orientation mechanism for the sex trivalent by the autosomal (pseudo)trivalents too. This protects the heterozygote pairs from the pressure of meiotic drive.

As a later event, cascades of reciprocal centromeric translocations between pre-existent metacentrics might produce further racial evolution (cf. Halkka et al. 1987). Translocation cascades can be constructed both in West-European and East-European phylogenetic groups. Racial mixing occurs inside the buffer zones (Fedyk 1986, Fedyk et al. 1991, Hausser 1991, Searle eg. in 1986, Wojcik 1993).

The chromosomal and racial polymorphism of *Sorex araneus* have evolved during and after the last glaciation. Most probably arm exchange processes and chromosomal evolution are occurring also in our time. We believe that the highly GC-rich centromeric heterochromatin plays an important role in the Robertsonian rearrangements producing new chromosomal polymorphism.

Acknowledgements. We thank Mrs Veronica Söderlund and Mrs Arja Ikävalko and for their help in photography. and Oskar Öflund Foundation for financial aid.

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