Molecular diversity of some *Crocidura* species (Insectivora, Soricidae) from Ethiopia

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Introduction

The rich and unique fauna of Ethiopia is associated with its extremely diverse geomorphology. At present, 277 mammalian species have been recorded from Ethiopia, 29 of which (10.5%) are endemic (YALDEN *et al.*, 1996). The small mammal fauna is particularly diverse. The family Soricidae is represented by 25 native species in Ethiopia, including 8 (32%) endemic ones. Local endemism may be rather high: of the 6 species known from the Bale Massif, 4 are endemic (HUTTERER and YALDEN, 1990). In view of the rapid habitat destruction in Ethiopia, taxonomic and evolutionary studies on Ethiopian small mammals are particularly important and urgent for identifying specific field conservation projects that are needed for endangered species. To improve our knowledge on the taxonomy, biodiversity, and phylogenetic relationships of Ethiopian shrews, we conducted a study using morphological, cytogenetical and DNA analyses. This paper presents a comparative study of repetitive DNA sequences in six species of shrews of the genus *Crocidura* from Ethiopia.

Material and methods

Species studied

All samples of shrews were collected by one of us (L. A. L.) during extensive trapping sessions in the framework of the Joint Ethio-Russian Biological Expedition (JERBE), from 1995 to 1998. The identification of specimens was kindly performed by Dr. R. Hutterer (Museum Alexander Koenig, Bonn). The specimens are deposited in the Zoological Museum of the Lomonosov Moscow State University (ZMMU). The localities of specimens used in this study are shown in table 1.

Species	n	Museum's catalogue numbers	Locality
C. glassi Heim de Balsac, 1966	3	S-164856 S-164853 S-164860	Eastern Plateau, Bale Mountains: mosaic grassland/forest habitats in the Dinsho area (3,170 m ASL, 7° 06′ N 39°47′ E)
C. thalia Dippenaar, 1980	3	S-165166 S-165165 S-164858	Eastern Plateau, Bale Mountains: Shawe River at *Aningeria* belt of the Harenna Forest (1,935 m ASL, 06° 38′ N 39° 44′ E)
Crocidura sp. B	2	S-165342 S-165343	Western Plateau: riverine variant of the humid afromontane Beletta Forest (1,900 m ASL, 07° 34′ N 36° 31′ E)
C. olivieri Lesson, 1827	1	S-166027	Western Plateau: *Acacia-Terminalia-Combretum* savanna and woodlands at the Middle Godjeb Valley (1,220 m ASL, 07° 15′ N 36° 47′ E)
C. macmillani Dollman, 1915	2	S-166029 S-166031	Western Plateau: *Acacia-Terminalia-Combretum* savanna and woodlands at the Middle Godjeb Valley (1,220 m ASL, 07° 15′ N 36° 47′ E)
C. parvipes Osgood, 1910	4	S-166030 S-166032 S-166033 S-166034	Western Plateau: *Acacia-Terminalia-Combretum* savanna and woodlands at the Middle Godjeb Valley (1,220 m ASL, 07° 15′ N 36° 47′ E)

Table 1
List of *Crocidura* shrew specimens used in the present study (n-number of specimens).

Crocidura glassi, *C. thalia* and *C. macmillani* are very similar in their morphology and were previously lumped under *C. fumosa* Thomas, 1904 (YALDEN *et al.*, 1976). *Crocidura olivieri* is morphologically very distinct, characterized by a large size. *Crocidura* sp. B is certainly a species new to science. A full description, diagnosis and nomenclature of this new species endemic to Ethiopia will be published elsewhere (LAVRENCHENKO and HUTTERER, in prep.).

The karyotype of all the species studied have been described. *Crocidura thalia*, *C. glassi* and *Crocidura* sp. B have 36 chromosomes (LAVRENCHENKO *et al.*, 1997; ANISKIN *et al.*, 1998) while *C. olivieri* and *C. parvipes* have 50 chromosomes. The diploid number of *C. macmillani* is 28 chromosomes. A detailed description of the karyotypes of these species will be published elsewhere (LAVRENCHENKO and HUTTERER, in prep.).

All these shrew species occupy different habitats on both sides of the Ethiopian Rift Valley. *Crocidura glassi* is a specialized moorland form, whereas *C. thalia* is a species of mountain grassland. *Crocidura* sp. B is a true forest species while *C. macmillani* and *C. parvipes* are inhabitants of woodlands and savannas. *Crocidura olivieri* is widely distributed throughout savanna and grassland habitats.

Restriction DNA analysis

Total DNA was extracted from ethanol fixed liver, testes and muscles by proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation according to a standard procedure (ARRIGHI *et al.*, 1968). We examined genomic DNA patterns of shrews with a specially optimized method of DNA repeated units detection called taxonomic DNA fingerprint or taxonprint (GRECHKO *et al.*, 1997; FEDOROV *et al.*, 1999). The difference between this approach and the traditional restriction DNA analysis (RFLP) is that we used short-cutting restriction endonucleases followed by phosphorous 32 end-labeling of the restriction fragments. This procedure has permitted to detect short fragments (30-300 bp) of repeated DNA sequences. The advantage of taxonprint analysis as a phylogenetic tool was described by GRECHKO *et al.* (1997) and FEDOROV *et al.* (1999). The main property of this approach is the intraspecific identity of band patterns, which enables to use small numbers of individuals (even only one

Taxon	Status of character
C. thalia	00000000000100010111000000001000110000000011011000000011111001110100000000001101001001111111111100101110
C. glassi	00000000000100010111001101001000110000000011011000000001110011101110111111001101001001010111100000101101
Crocidura sp. B	00000000010100010111000010011000111000000011011010000000111001110100000000001101001001011111110010101110
C. olivieri	01100010100011010101100000101110110010000110110000000000011111111001000000111111011011000110000000000100
C. macmillani	00000000000000010101000000010001100000001000000100000000100111010000000000011010010010001100000000000100
C. parvipes	10010100001000010101110000100000110000110010000100011100001001110100000000101111011010001100000000000100
S. murinus	00001001000000101101000000000011101010010100000001000000000010100000000000010010010000001100000010101000

Table 2
Complete character matrix for phylogenetic analysis of six *Crocidura* species and *Suncus murinus* as outgroup for MspI, HinfI, TaqI, BcnI and Eco130I restriction endonucleases. Total number of bands is 105.

for rare species) and does not require any intraspecific statistical analysis. Another property of electrophoretic bands of DNA repeats revealed by our technique is the hierarchical order of their organization which can be seen on a higher taxonomical level: bands can be common for a group of related species such as genus and family (BANNIKOVA *et al.*, 1996; FEDOROV *et al.*, 1999).

Conditions of experiment

Five restriction enzymes were used: MspI, HinfI, TaqI, BcnI, Eco130I. 0.5 microgram genomic DNA was incubated with 5 U of restriction endonuclease in 20 µl of its specific buffer for 4-12 h. Labeling the recessed 3′ termini of DNA restriction fragments (0.1 µg) was carried out in 10 µl of buffer (10 mM Tris-HCl /pH 7.5/, 10 mM $MgCl_2$, 50 mM NaCl) in the presence of 1 µCi of appropriate [α^{32}P]dNTP, 20 µm of each of the three remaining cold dNTPs, and 0.5 U of Klenow fragment for 15 min at 20 °C. The reaction was stopped by the addition of EDTA to 10 mM. Samples (2-3 µl) were subjected to electrophoresis in non-denaturing 10% polyacrylamide gels with

	C. thalia	*C. glassi*	*C. olivieri*	*Crocidura* sp. B	*C. macmillani*	*C. parvipes*
C. glassi	0,238					
C. olivieri	0,494	0,517				
Crocidura sp. B	0,136	0,247	0,500			
C. macmillani	0,344	0,385	0,375	0,355		
C. parvipes	0,500	0,525	0,418	0,506	0,333	
Suncus murinus	0,594	0,618	0,612	0,600	0,422	0,567

Table 3
Matrix of genetic distances according to the method of Nei and Li (1979).

1xTBE buffer at 800-1200 V for 4-5 h. After electrophoresis the gel was dried and autoradiographed for 16-48 h. pBR322 DNA fragments digested with MspI were used as molecular-weight markers.

Phylogenetic analysis

The number and distribution of bands corresponding to digested DNA fractions were examined in autoradiographs of the species tested. The lack or presence of a band with equal electrophoretical mobility was designated as "0" and "1", respectively. Thus the results of experiments with all endonucleases were presented in the form of a matrix composed of 0/1, reflecting the state of binary characters (tabl. 2). On the whole, 105 bands were detected across the 7 species tested, including the outgroup. This data set includes 92 variable characters, 26 of which are informative; 13 bands were common for all tested species. Phylogenetic analyses with *Suncus murinus* as an outgroup were carried out using neighbour-joining (NJ) and parsimony methods in TREECONW (VAN DE PEER and DE WACHTER, 1994) and Phylip version 3.5 (FELSENSTEIN, 1993) packages, respectively. Bootstrap analysis with 500 replicates was conducted to assess the reliability of branching patterns. The genetic distances in NJ tree based on a total of 105 characters were computed by the formula of NEI and LI (1979) (tabl. 3).

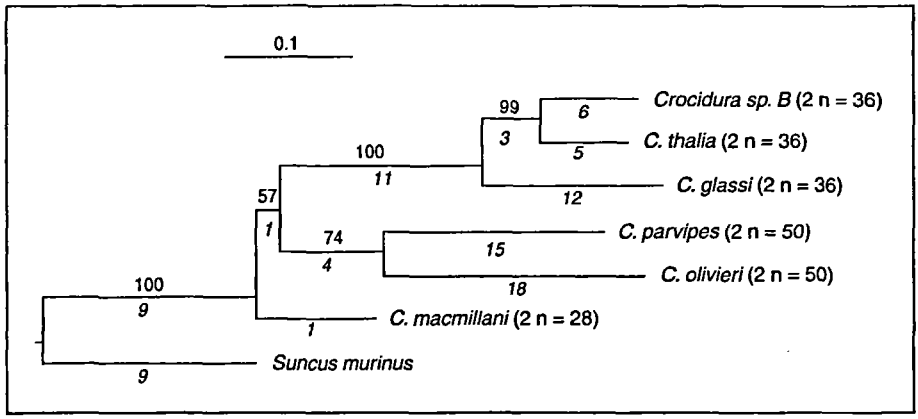
Results and discussion

The results of restriction nuclease analysis are shown in the 0/1 matrix (tabl. 2), in the table of genetic distances (tabl. 3) and on the NJ dendrogram (fig. 1) The topologies based on both NJ and parsimony methods appeared similar. Here we present only the NJ tree, which branch lengths correspond to genetic distances (following NEI and LI, 1979). The standard indicators of support of the inferred tree topology are high (consistency indices with uninformative characters included or excluded of 0.979 and 0.929, respectively).

According to our data the 36 chromosome species belong to the same monophyletic cluster with *Crocidura* sp. B and *C. thalia* being more closely related to each other than either is to *C. glassi*, which itself is equally divergent from *Crocidura* sp. B and *C. thalia*. *Crocidura olivieri* and *C. parvipes* joined into the same phyletic lineage. The 50 chromosome species and 36-chromosomal species appear to constitute a sister group of *C. macmillani* which forms a basal branch in both reconstructions, with poor bootstrap support.

Analysis of DNA divergence degree in different species was carried out by length comparison of appropriate branches of dendrogram. Both methods indicate strong differences in rates of DNA evolution between *C. macmillani* and other taxa. To confirm this hypothesis a modification of the relative rate test (SARICH and WILSON, 1967) was applied. The level of divergence from the outgroup of each terminal taxon was assessed as the fraction of bands (out of 105 characters) found in a given species but lacking in *Suncus murinus*. The comparison of estimates obtained on the basis of 2000 bootstrap replicates showed that *C. macmillani* is significantly less divergent ($p < 0.01$) from the outgroup than any other species studied. The relative rate test by TAKEZAKI *et al.* (1995) in the Phyltest program, version 2.0 (Kumar, 1995), was also applied. The difference of evolutionary rate in the lineage of *C. macmillani* is statistically significant, and the rate constancy is rejected at 5% level ($z = 8.694$).

Conversely, there is no significant difference of evolutionary rates between lineages of 36 and 50 chromosome species ($z = 0.224$). However, the differences accumulated along the stem of the 36 chromosome group is greater by a factor of 2.7. Eleven potential synapo-



I Figure 1
Neighbour joining dendrogram of the relationships of six *Crocidura* shrews from Ethiopia. Length of branches corresponds to Nei and Li (1979) genetic distances. The numbers under a line correspond to the number of changes observed along the branches of the maximum parsimony tree. Numbers above the branches show bootsrap support (% of 500 replications). *Suncus murinus* is used as an outgroup.

morphies (band gains) are revealed for the clade comprising the three 36 chromosome species while there are only 4 bands unique for the *parvipes-olivieri* clade (the difference is nearly significant, $p > 0.06$, Fisher criterion). If we assume a clocklike evolution for this part of the tree, the results can be regarded as an indication of a relatively recent divergence within the 36 chromosome clade. *Crocidura glassi* and *C. olivieri* have accumulated more changes than the other taxa while *C. macmillani* has acquired fewer differences.

Therefore the 36 chromosome species, 50 chromosome species and 28 chromosome *C. macmillani* form distinct phylogenetic lineages. It is clear that DNA repeated sequences evolution correlates well with chromosome number divergence of the species studied but not directly with morphological diversity. It is essential to note that except for *C. macmillani*, all cytogenetically studied endemic Ethiopian *Crocidura* species (including *C. bottegoides* HUTTERER and YALDEN, 1990; *C. harenna* HUTTERER and YALDEN, 1990; *C. lucina* DIPPENAAR, 1980) have 36 chromosomes (LAVRENCHENKO *et al.*, 1997). Although African *Crocidura* species are extremely numerous

and diverse (morphologically and cytogenetically), only two species with diploid numbers of 36 were found in the rest of Sub-saharan Africa (*C. obscurior* from Ivory Coast and *C. luna* from Burundi) (SCHLITTER *et al.*, 1999). Taking into account this evidence along with the results of our phylogenetic analysis, we may suppose that all 36 chromosomal species endemic to Ethiopia form a monophyletic group of a relatively recent origin.

DIPPENAAR and MEESTER (1989) believed that the five Ethiopian taxa (*C. macmillani*, *C. baileyi*, *C. glassi*, *C. lucina* and *C. thalia*) form a distinct group of closely related endemic species. Our phylogenetic analysis demonstrated that *C. macmillani* is phyletically distant from the group comprising the other Ethiopian endemics, although a moderate bootstrap value supports this branching pattern. The topology of the tree is congruent with chromosomal data. The former species can be hypothesized to be similar to the ancestor of at least several lineages of African *Crocidura*.

The essential differences among the species examined were reflected in DNA repeated units patterns, confirming that the Ethiopian *Crocidura* species form a group with a high level of both morphological and molecular divergence. Our data suggest that the fauna of Ethiopian endemic *Crocidura* has a complex origin and comprises both an ancient lineage and a group of closely related species that has undergone recent (and presumably adaptive) radiation.

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