Russian Journal of Genetics, Vol. 38, No. 6, 2002, pp. 714–724. Translated from Genetika, Vol. 38, No. 6, 2002, pp. 853–864.
Original Russian Text Copyright © 2002 by Bannikova, Matveev, Kramerov.

Using Inter-SINE–PCR to Study Mammalian Phylogeny

A. A. Bannikova[1], V. A. Matveev[1], and D. A. Kramerov[2]

[1] *Department of Vertebrate Zoology, Moscow State University, Moscow, 119899 Russia; e-mail: grechko@genome.eimb.relarn.ru*

[2] *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, 119991 Russia*

Received October 31, 2001

Abstract—Results of the use of the fingerprinting method related to short interspersed elements (SINEs), inter-SINE–PCR, in the study of phylogenetic and taxonomic relationship in mammals from orders Chiroptera (family Vespertilionidae) and Lipotyphla (family Erinaceidae) are reported. The inter-SINE–PCR method is based on the amplification of fragments situated between copies of SINEs, which are short retroposons spaced 100 to 1000 bp apart. Specifically selected primers were used, which are complementary to consensus sequences of two short retroposons: the mammalian interspersed repeat (MIR), which is typical of all mammals and some other vertebrates, was used in the cases of bats and Erinaceidae, and the ERI-1 element recently isolated from the genome of the Daurian hedgehog was used in the case of Erinaceidae. The results support the current view on phylogenetic relationship between hedgehogs belonging to genera *Erinaceus, Hemiechinus*, and *Paraechinus* (but not the genus *Atelerix*). In bats, the phylogenetic reconstruction revealed a statistically valid topology only at lower taxonomic levels, whereas the topology for the genus and supragenus ranks was unresolved and fan-shaped. The benefits and limitations of the inter-SINE–PCR method are discussed.

INTRODUCTION

In modern studies on the phylogeny and taxonomy of the intraorder level, the role of PCR-based molecular markers has significantly enhanced. The examples are molecular markers obtained using the following methods: RAPD, which detects randomly amplified polymorphic DNA fragments in PCR with a single arbitrary primer [1]; SSR–PCR, which enables one to amplify DNA fragments situated between microsatellites [2]; and AFLP, which represents the results of selective amplification of restriction fragments of genomic DNA and combines the potentialities of RFLP and PCR [3]. The search for universal markers that are applicable for both diagnostic and phylogenetic purposes is continued.

In this work, we report the results of using one more variety of fingerprinting, which is related to short interspersed elements (SINEs). We used this method, inter-SINE–PCR, in the studies of taxonomic relationship in mammals. SINEs (short interspersed elements, short retroposons) are repeated 80- to 400-bp DNA sequences, tens and even hundreds of thousands copies of which are dispersed throughout the genome [4–6]. SINEs are transcribed by RNA polymerase III, and the reverse transcription of the newly formed low-molecular-weight RNA can result in the formation of new SINE copies. The overwhelming majority of SINE families originate from tRNA molecules, which was established from the similarity between the nucleotide sequences of tRNA and the head part of SINEs. The tail part of SINEs is usually formed by an A-rich sequence, whereas the central part is quite different in different SINE families. The presence or absence of a SINE family in a genome is a perfect synapomorphy, because, once appeared, the SINE family is preserved in all later separated branches of the given stem of the evolutionary tree [7, 8]. Another perfect synapomorphy is the presence or absence of some SINE copies in certain loci of the genome. These traits were successfully used in phylogenetic studies of mammals and fish [9, 10]. However, this approach is labor-consuming and expensive.

Another approach to phylogenetic studies that is also based on using SINEs is considerably easier and less expensive. This method, inter-SINE–PCR, consists in the amplification of fragments situated between SINE copies situated at a relatively small (100 to 1000 bp) distance apart (Fig. 1). For the first time, restriction polymorphism of DNA fragments flanked by short interspersed repeats was investigated in the study of *Alu*-polymorphism in the human genome (so-called *Alu*–PCR or IRS–PCR [11]). Later, polymorphism of other short interspersed repeats was studied in the mammalian order Artiodactyla [12]

Our attention was drawn by a SINE family named the mammalian interspersed repeat (MIR), which is represented by 10^5 copies probably in all mammalian genomes [13–15]. The MIR elements represent one of the most ancient of known SINE families, which is indicated by their wide distribution and an extremely high (up to 50%) divergence of their copies. The latter fact also indicates that, in contrast to other known SINEs, MIR stopped multiplying long ago. Apparently,

1022-7954/02/3806-0714$27.00 © 2002 MAIK “Nauka / Interperiodica”

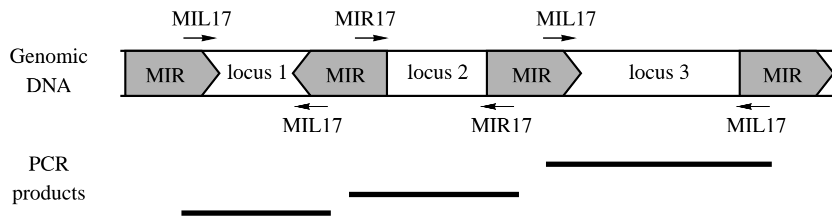

Fig. 1. Scheme of SINE–PCR. Four MIR element copies are shown, of which three are oriented in one direction and one in the other. Thin arrows indicate primers MIL17 and MIR17, which are complementary to different DNA strands. Taken separately, each of the primers ensures the amplification of the regions situated between tail-to-tail- or head-to-head-oriented MIR element copies (locus 1 or locus 2, respectively). Simultaneous presence of both primers in the reaction mixture ensures the amplification of the fragment flanked by equally (tail-to-head) oriented MIR element copies (locus 3).

because of the ancient origin, full-sized MIR copies (260 bp in length) occur relatively seldom. As a rule, the copies are lacking one or both termini, and the majority of these copies are represented by the only central region (core sequence), 70 bp in length, for which the reasons are not clearly understood. Differences in the patterns of inter-SINE–PCR products are caused by both mutations in the copies of these repeats per se and deletions and insertions in DNA regions situated between the copies.

To date, the inter-SINE–PCR method was used only for the studies of phylogeny in Artiodactyla [16]. The objects of our study were mammals belonging to two orders: Lipotyphla and Chiroptera. In addition to the MIR elements, we studied a short retroposon ERI1 (158 bp in length), which is specific of only the family Erinaceidae and has recently been isolated from the genome of the Daurian hedgehog *Hemiechinus dauuricus* [17]. Note that such SINEs, which are characterized by a narrow distribution range and a relatively low divergence of copies, were never used in phylogenetic studies conducted by the inter-SINE–PCR method before our work.

According to recent data of molecular macrotaxonomy [18, 19], true insectivorous mammals Eulipotyphla and bats Microchiroptera represent a monophyletic group, whereas they greatly differ in the direction of radiation. Ancient insectivorous mammals were a very generalized group, which allowed them to become ancestral forms for most of latest placental animals. From their origin till the present, Chiroptera have been related to the most specialized mammals, while some of them, e.g., bats, preserved some particular morphological features that bring these bats closer to insectivorous mammals [20]. Among Lipotyphla, the most ancient family is Erinaceidae. So far, no consensus exist on the number of genera and species of this family. The largest bat family, Vespertilionidae, is also the most taxonomically confused. All of the above has motivated a comparative study of these two taxa.

MATERIALS AND METHODS

Samples. In Lipotyphla, we examined 39 hedgehogs (subfamily Erinaceinae) and 2 gymnures (subfamily Hylomyinae); in Chiroptera, 40 vespertilionid bats (family Vespertilionidae), 2 bats of different horseshoe bat species (family Rhinolophidae), and 2 bulldog bats (family Molossidae). The description of samples is given in Table 1.

DNA isolation. DNA was isolated from ethanol-fixed tissues (muscles, testes, kidneys, and liver) by phenol–chloroform extraction following the treatment of tissue homogenates with pronase [21].

Conditions of inter-SINE–PCR. Inter-MIR–PCR (hereafter, MIR–PCR) was conducted using one or two primers that were complementary to the most conserved region of the central core sequence of the MIR element [14]: MIR17, 5'-AGTGACTTGCTCAAGGT-3', and MIL17, 5'-GCCTCAGTTTCCTCATC-3' (Fig. 1). Primers (100 pmole each) were labeled with [γ^{32}P]-ATP (1 MBq) by polynucleotide kinase [22]. PCR was conducted in 20 μl of a reaction mixture containing 10 mM of a Tris–HCl buffer, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 0.001% gelatin; dNTPs, 0.2 mM each; 4 pmole of each primer; 1 EU of *Taq* polymerase (Sileks); and 25 ng of DNA template. The conditions of MIR-specific PCR corresponded to an earlier described schedule [14]: denaturation, 30 s at 94°C; annealing, 45 s at 56°C; and elongation, 2 min at 72°C. The number of cycles was 27. The initial denaturation and final synthesis lasted for 3 min at 94°C and 5 min at 72°C, respectively.

In ERI–PCR, we used the primer that was complementary to the fore end of the ERI1 element (5'-CCACCTCCC(G/A)GGACCAC-3') [17] and initiated DNA synthesis in the direction upstream of the SINE. The primer was labeled as described above. In the experiments with ERI1, a consistently reproducible pattern was obtained upon increasing the annealing temperature by 5°C as against the calculated temperature. As a result, PCR was conducted under the following conditions: denaturation, 30 s at 94°C; annealing, 45 s at 65°C; and elongation, 2 min at 72°C. The number of cycles was 27. The initial denaturation and final synthesis lasted for 3 min at 94°C and 5 min at 72°C, respectively.

All PCR procedures were conducted in an MJ Research thermal cycler (MJ Research, United States). PCR products were denaturated and separated by elec-

Table 1. Description of material

Sample no.	Species	Number of animals studied	Localities
			Lipothyphla
1–14	*Erinaceus roumanicus*	14	Kaluga oblast, Ryazan' oblast (Solotcha), Moscow oblast (Nikolina Gora), Bryansk oblast (Katalino and Vyshkov), Stavropol' krai, Kabardino-Balkaria (Nal'chik), and Dagestan
15–21	*E. concolor*	7	Azerbaijan (Lenkoran'), Abkhazia (Gudauty), Turkey*, and Israel (Haifa)*
22–27	*E. europaeus*	6	Moscow oblast (Nikolina Gora and Krasnogorsk), Tver' oblast (Kolchevatiki and Krutitsy), and United Kingdom (Norwich)*
28	*E. amurensis*	1	Primorskii krai (Ussuriisk raion)
29	*Atelerix frontalis*	1	South Africa**
30	*A. algirus*	1	Gran-Canario (Las Palmas)
31	*A. albiventris*	1	Senegal (Dakar)*
32–33	*Paraechinus aethiopicus*	2	Saudi Arabia (Oumamah)*, Saudi Arabia**
34	*P. hypomelas*	1	Turkmenistan
35	*Hemiechinus dauuricus*	1	Buryatia
36–37	*H. auritus*	4	Turkmenistan (Kara-Kala and Babarab) and Stavropol' krai (Stepanovskoe)
40	*Neotetracus sinensis*	1	Vietnam***
41	*Hylomys suillus*	1	Thailand (Chaiyaphum)**
			Chiroptera
1–3	*Pipistrellus nathusii*	3	Moscow oblast and Byelorussia
4	*P. pipistrellus*	1	Krasnodar krai (Tuapse raion)
5–6	*P. kuhli*	2	Volgograd oblast (Mikhailovskii raion)
7	*P. abramus*		Vietnam (Hanoi)
8	*Hypsugo pulveratus*	1	Vietnam (province Binh Quan)
9	*H. savii*	1	Primorskii krai
10	*Nyctalus noctula*	1	Volgograd oblast (Mikhailovskii raion)
11–12	*N. leisleri*	2	Volgograd oblast (Mikhailovskii raion)
13	*Vespertilio sinensis*	1	Chita oblast (Daurian Reserve)
14	*V. murinus*	1	Obtained from captivity
15	*Eptesicus serotinus*	1	Kabardino-Balkaria (Nal'chik)
16–17	*Scotomanes ornatus*	2	Nepal (Annapurna), Vietnam
18	*Hesperoptenus blanfordi*	1	Cambodia (Phnom Bokor)
19	*Miniopterus schreibersii*	1	Nepal (Annapurna)
20–21	*M. sp.*	2	Cambodia (Phnom Bokor)
22	*M. inflatus*	1	Ethiopia (province Bale)
23	*Plecotus auritus*	1	Volgograd oblast (Mikhailovskii raion)
24	*P. balensis*	1	Ethiopia (province Bale)
25	*Barbastella leucomelas*	1	Tajikistan (Khujand)
26	*Myotis montivagus*	1	Vietnam
27	*M. annamiticus*	1	Vietnam (province Binh Quan)
28–29	*M. daubentoni*	2	Moscow oblast (Tuchkovo)
30–32	*M. muricola*	3	Cambodia (Phnom Penh and Kampot) and Nepal
33	*M. csorbai*	1	Nepal (Annapurna)
34	*M. ikonnikovi*	1	Primorskii krai (Ussuriisk raion)
35	*M. nattereri*	1	Moscow oblast (Tuchkovo)
36	*M. blythi*	1	Georgia
37	*M. dasycneme*	1	Moscow oblast (Tuchkovo)
38	*M. brandti*	1	Moscow oblast (Tuchkovo)
39	*M. mystacinus*	1	Krasnodar krai (Tuapse raion)
40	*Harpiocephalus mordax*	1	Cambodia (Phnom Bokor)
41	*R. ferrumequinum*	1	Krasnodar krai (Adler raion)
42	*Rhinolophus affinis*	1	Ethiopia (province Bale)
43–44	*Chaerephon plicata*	2	Cambodia (Phnom Bokor)

* The material for DNA isolation was kindly presented by F. Santucci (School of Biological Sciences, University of East Anglia, Norwich, UK).

** The material for DNA isolation was kindly presented by F.M. Catzeflis (Institut des Sciences de *l'Evolution, Universit* Montpellier, France).

*** The material for DNA isolation was collected by G.V. Kuznetsov (Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences).

trophoresis in a 6% polyacrylamide gel containing a Tris–borate buffer and 8M urea (similar to a sequencing gel composition). Gel length and width were 50 cm and 0.4 mm, respectively. Electrophoresis was run for 7 h at a constant power capacity of 75 W. Dried gels were autoradiographed by gel exposure with a Retina X-ray film for 16 to 48 h.

Phylogenetic analysis. The data from fingerprints obtained by MIR– and ERI–PCR, were compiled into a binary matrix (where 1 or 0 are the presence or absence of the marker, respectively), which was analyzed by the Wagner maximum parsimony (MP) method (PAUP software package, version 4.0b4a [23]), and by the neighbor joining (NJ) method (TREECONW software package [24]). Genetic distances (D_L) were calculated according to Link *et al.* [25] and related to Jacquard's similarity index (J) by the following formula: $D_L = 1 - J$. The Bootstrap analysis included 1000 iterations.

RESULTS AND DISCUSSION

Description of Primers and Characterization of Inter-SINE–PCR Patterns

In all studied species of Erinaceidae and Chiroptera, we obtained highly informative patterns of PCR DNA products that contained many clearly visible bands. As an example, Fig. 2 shows a fragment of an electrophoregram containing the MIR–PCR products in bats. The top part (approximately, one-eighth) of the electrophoregram, which contains fragments with a length of more than 1000 nucleotides (nt), was not analyzed and is thus omitted from Fig. 2.

MIR copies are present in the genome in both orientations. This provides the possibility of using either one or two oppositely directed primers in the MIR-specific PCR (Fig. 1). In the case of one primer, only oppositely (head-to-head or tail-to-tail) oriented MIR copies are amplified. In the case of primer MIL17, which initiates the amplification of loci flanked by tail-to-tail–oriented MIR copies, 100 to 200 fragments with a length of 100 to 1000 bp were detected in each bat DNA sample. In hedgehogs, a relatively small number of fragments was amplified using this primer (no more than 40 fragments per sample). To increase the number of fragments in hedgehogs, we used one more MIR-specific primer, MIR17, which initiates the amplification of loci flanked by head-to-head–oriented MIR copies. The concurrent use of these to oppositely directed primers enabled us to increase the number of fragments and to obtain smaller fragments (90 to 1500 nt in length). In this case, hedgehog fingerprints contained 60 to 70 bands in each DNA sample. Apparently, this is explained by the fact that PCR with two primers proceeds on both oppositely and identically (head-to-tail) directed copies (Fig. 1).

In hedgehogs, we analyzed fingerprints obtained by MIR–PCR with two primers. For 40 hedgehog DNA samples, the number of binary characters taken into consideration was 298. For 44 bat DNA samples, the number of binary characters taken into consideration was 757. Since we obtained so rich a pattern, we considered it inexpedient to increase it by introduction of the second primer.

We found that, in ERI-specific PCR, the ERI1-specific primer sequence must be complementary to the head part of the repeat. In this case, the amplification proceeds on oppositely directed (head-to-head) copies. In the attempts of using primers complementary to the central region of the retroposon, the amplification of tail-to-tail-oriented copies proceeded less successfully. In this case, PCR patterns consisted of unclear, blurred bands. We suppose that this was caused by the formation of hairpin structures because of adhesion between long complementary regions, which are available in the termini of PCR products in this case. Moreover, in this variant, not only the region that is flanked by the retroposon but also long regions of the repeat itself are amplified. Therefore, the PCR products contain many fragments that are too long to be qualitatively analyzed. In ERI–PCR with one primer, fragments with a length of 70 to 700 nt were detected: 50 fragments in hedgehogs and 18 to 20 fragments in gymnures. The number of binary traits taken into consideration was 237 for 41 samples. As in the case of MIR–PCR, fragments of more than 700 nt were omitted from analysis.

Figures 3–5 show phylogenetic trees constructed using the NJ method. As the topology of the NJ and MP dendrograms was identical, the MP trees are not presented.

Individual and Geographical Variation

In this work, we take individual variation to mean intrapopulation variation over a limited area, for example, within the boundaries of oblast or raion.

MIR–PCR appeared to be more sensitive in the detection of individual variation than ERI–PCR. The MIR–PCR patterns of all studied samples were characterized by individual variation. Only three samples of East-European hedgehogs from Vyshkov, Bryansk oblast, were exceptions: they had identical patterns. For samples from Bryansk oblast, genetic distance (D_L) was 0.00 to 0.09; for samples from Stavropol' krai, 0.02 to 0.09; for samples from Abkhasia, 0.02 to 0.10. The samples of *E. europaeus* from two localities of Moscow oblast (Nikolina Gora and Krasnogorsk) appeared to be relatively distant from each other ($D_L = 0.13$) (Fig. 3).

As a rule, fingerprints of individuals from geographically distant localities were more different from one another than fingerprints of individuals from the same locality. Apparently, such variation is related to the interpopulation level. In most cases, D_L between hedgehogs of the same species from geographically distant localities varied from 0.1 to 0.35. However, genetic distance between individuals is not directly related to geographical proximity of localities. Thus, an East-Euro-

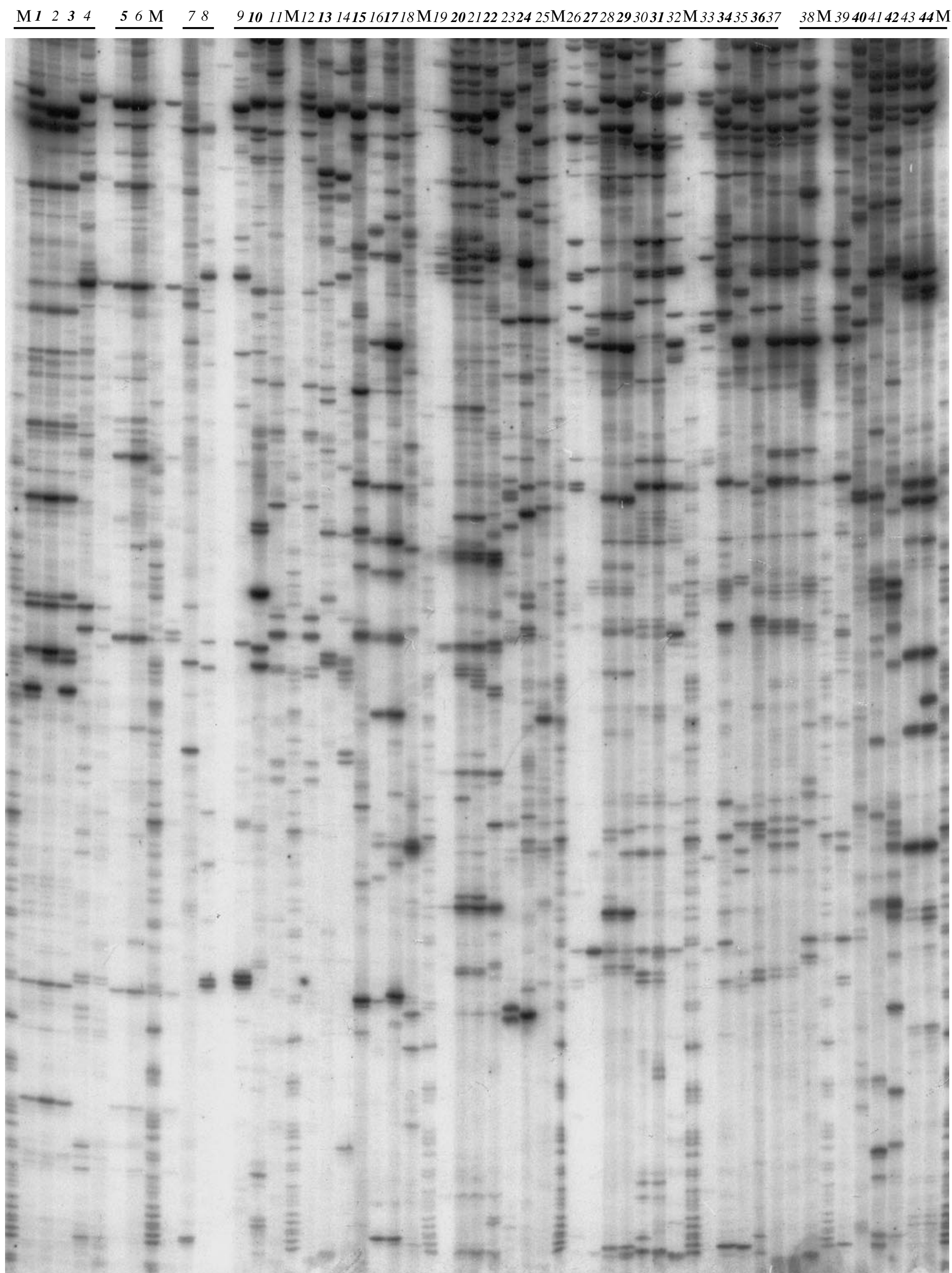

Fig. 2. Electrophoretic fractioning of MIR–PCR products of genomic DNA in 31 bat species. Lanes correspond to the order of species listed in Table 1. M is a marker representing the product of sequencing of plasmid pGEM7z (for one out of four nucleotides). The markers were added once in each set of seven or eight lanes.

pean hedgehog from Moscow oblast was included in different clusters with hedgehogs from geographically close localities (Kaluga, Ryazan'), and hedgehogs from Stavropol' krai unexpectedly fell into one cluster with hedgehogs from Bryansk oblast rather than with hedgehogs from Nal'chik and Dagestan, which belong to the same region. West-European hedgehogs from Moscow and Tver' oblast were very closely related (D_L = 0.02–0.08) and did not form any regional groups. Long-eared hedgehogs from Stavropol' krai and eastern and western Kopet-Dag also were not clustered in accordance with their geographical proximity.

In bats, individual genetic distances varied over a wide range. A remarkable fact is that genetic distances for *Pipistrellus nathusii* individuals from three different European colonies in the same geographical locality

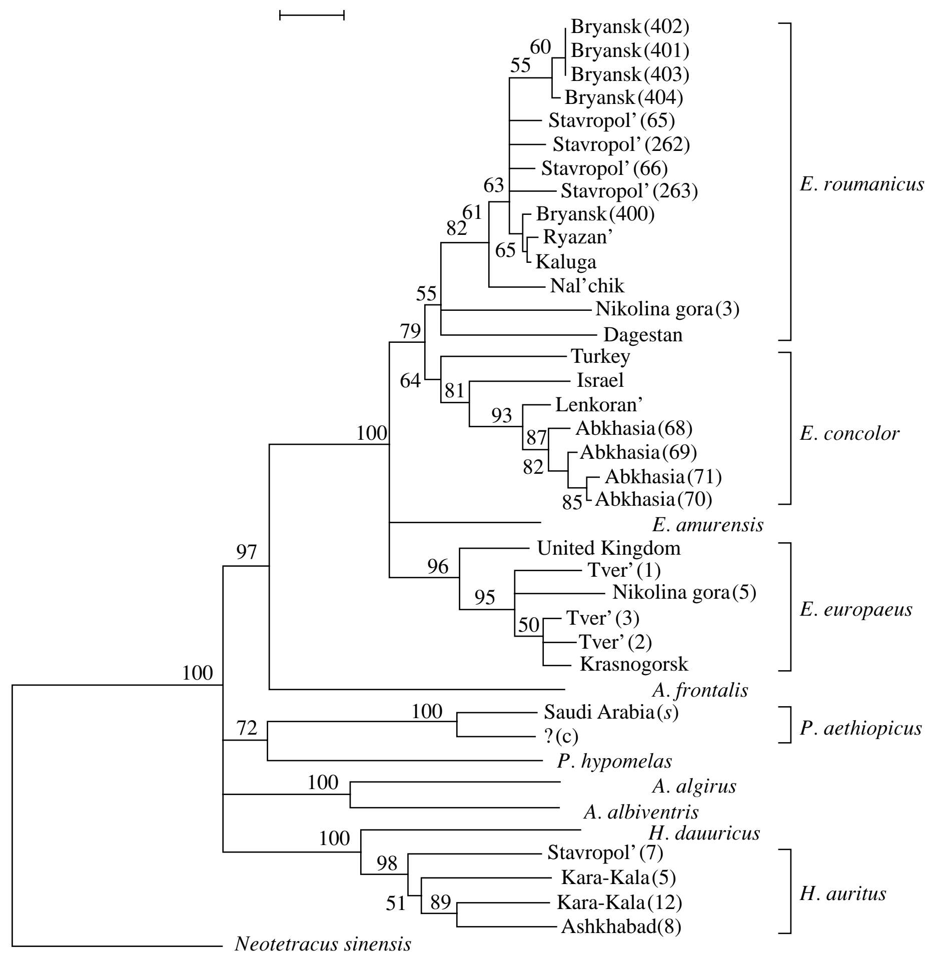

Fig. 3. NJ dendrogram of phylogenetic relationship in hedgehogs based on the MIR–PCR results. Numbers above the branches are bootstrap indices (percent of 1000 trials); clusters with a validity of less than 50% are shown unresolved and are not provided with bootstrap indices. Collection codes are given after sample localities in parentheses. The species *Neotetracus sinensis* was used as outgroup.

were not related to the remoteness of these colonies from one another. The genetic distance between two *P. nathusii* bats, one from Vitebsk oblast and the other from Moscow oblast, was only 0.24, whereas the distances between these bats and another *P. nathusii* bat belonging to another raion of Moscow oblast was 0.41. Two *Scotomanes ornatus* bats from very distant, isolated populations from Nepal and Vietnam were characterized by a very great genetic distance ($D_L = 0.51$); however, this genetic distance was lower than genetic distances for different species belonging to the same genus, for example, 0.85 for two *Myotis* species (*Myotis daubentoni* and *M. muricola*). A surprisingly great genetic distance was found between two *M. muricola* individual from Nepal and two *M. muricola* individuals from different regions of Cambodia ($D_L = 0.74$). This distance even exceeds some intraspecific genetic distances in other genera. Probably, these *M. muricola* samples should be related to different species. It is known that *M. muricola* is characterized by considerable variability of many morphological traits, and the taxonomy of the entire *Myotis* genus is considered by some authors only "preliminary" [26].

Minimum and maximum values of MIR–PCR-based D_L for hedgehogs and bats are shown in Table 2. As seen from Table 2, individual and geographical variation is distinctly greater in bats. In both hedgehogs and bats, a wide range of D_L variation characterizes geographical variation. In bats, this type of distance exhibited highest variation. In hedgehogs, the interspecific D_L distance varied in the greatest range. The results for both taxa indicate that a genetic distance calculated based on inter-SINE–PCR markers reflect not only the geographical distance between individuals or populations.

Taxonomic Variation and Phylogeny

Erinaceidae. As an outgroup, we used two gymnures, *Neotetracus sinensis* or *Hylomys suillus.* The replacement of one of these outgroups by the other did not fundamentally affect the tree topology or bootstrap

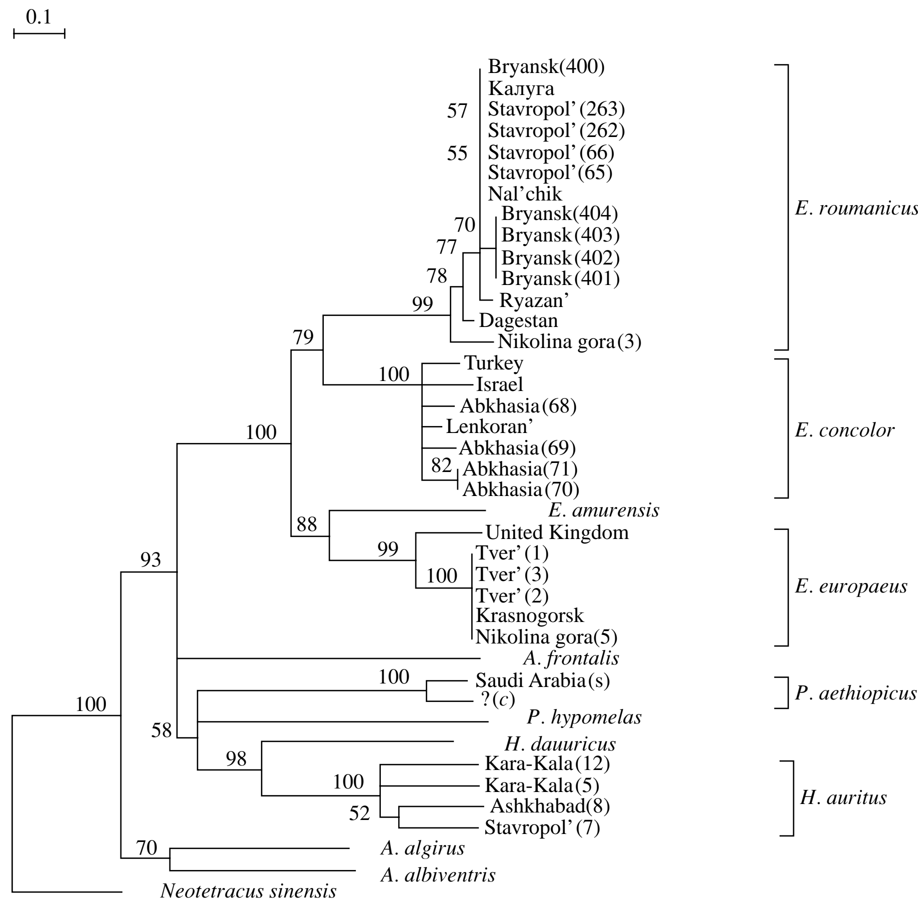

Fig. 4. NJ dendrogram of phylogenetic relationship in hedgehogs based on the ERI–PCR results. Designations as in Fig. 3.

indices (BI). The trees that were constructed based on MIR–and ERI–PCR differed in the number of resolved clusters and in the position of *Atelerix frontalis* and the *A. algirus–A. albiventris* cluster (Figs. 3 and 4).

In the MIR–PCR-based tree (Fig. 3), *A. frontalis* with a high BI (97%) is included in the group corresponding to the genus *Erinaceus.* Withing the genus *Erinaceus*, the clusters corresponding to *E. concolor* and *E. roumanicus* had very moderate BI (55 and 64%), and the position of *E. amurensis* in relation to this species pair and *E. europaeus* is uncertain (BI = 42%). The position of the *A. algirus–A. albiventris* cluster is actually unresolved, because its attachment to the genus *Paraechinus* was not supported by BI (34%). The same concerns the genus *Hemiechinus*: the position of this cluster on the same branch with genera *Paraechinus* and *Atelerix* was characterized by a moderate BI of only 47%.

In the ERI–PCR-based tree (Fig. 4), the hedgehog genera were better resolved, which made phylogenetic relationships among them more clear than in the case of MIR–PCR-based tree. The genus *Erinaceus* was subdivided into four clusters (BI = 90–100%), which correspond to four species. The position of *A. frontalis* is uncertain (BI = 47%). The position of the *A. algirus– A. albiventris* cluster is basal in relation to all other clusters (BI = 93%).

The species belonging to the genus *Erinaceus* deserve special attention, because the species status of forms *concolor* and *roumanicus* is debatable and far from being accepted by all taxonomists [27]. Our results confirm the assumption on a profound divergence between East-European hedgehogs from eastern Europe, on the one hand (form *roumanicus*), and from Asia Minor and the Near East, on the other (form *concolor*) [28, 29]. Apparently, these forms should be assigned the status of species.

Taxonomically, our results indicate the following: (1) The genus *Erinaceus* includes four species, because the subspecies forms *concolor* and *roumanicus* deserve the rank of a separate species. (2) The Daurian hedgehog does not deserve the rank of a separate genus, is related to the genus *Hemiechinus*, and is phylogenetically distant from *Erinaceus.* This result confirms the evidence obtained earlier based on another molecular marker [30]. (3) The assignement of Brandt's hedgehog (*Paraechinus*) into the same genus with the long-eared hedgehog (*Hemiechinus*) [31–33] is incorrect. (4) Of

African hedgehogs studied, the South-African species *Atelerix frontalis* is phylogenetically closer to the genus *Erinaceus*. This conclusion is the most unexpected. By morphological criteria, all three species of the genus *Atelerix* are rather close to each other and to the genus *Erinaceus*. Sometimes they are included in the latter genus [31, 34], but more often *Atelerix* is considered a separate genus phylogenetically close to *Erinaceus* [32, 33]. A viewpoint also exist on isolating of the South-African hedgehog *A. frontalis* and the Algerian hedgehog *A. algirus* into a genus *Aethechinus* [35, 36]; however, other authors do not accept it [31, 37]. Our results are in contradiction with either of these variants, because only two of these species were clustered together (*A. algirus–A. albiventris*), and only one of them, *A. frontalis*, was actually close to species belonging to the genus *Erinaceus*. Thus, as follows from our data, the South-African hedgehog represents one genus, and the Algerian and Cenral-African hedgehogs belong to another one.

Table 2. Genetic variation estimated based on the results of MIR–PCR in Erinaceidae and Chiroptera at different taxonomic levels

Taxonomic level of variation	D_L	
	Erinaceidae	Chiroptera
Intraspecific		
individual	0.0–0.13	0.2–0.33
geographical	0.1–0.35 (0.02)	0.3–0.5
Interspecific	0.35–0.7	0.7–0.86
Intergeneric	0.75–0.84	0.9–0.96 (0.81)

Note: The cases of deviation from means are given in parentheses.

Chiroptera. A distinctive feature of bat fingerprints was a high degree of specificity, i.e., the predominance of unique fragments that are species-specific or shared by species groups within the same genus over the fragments discriminating the related taxa having a higher

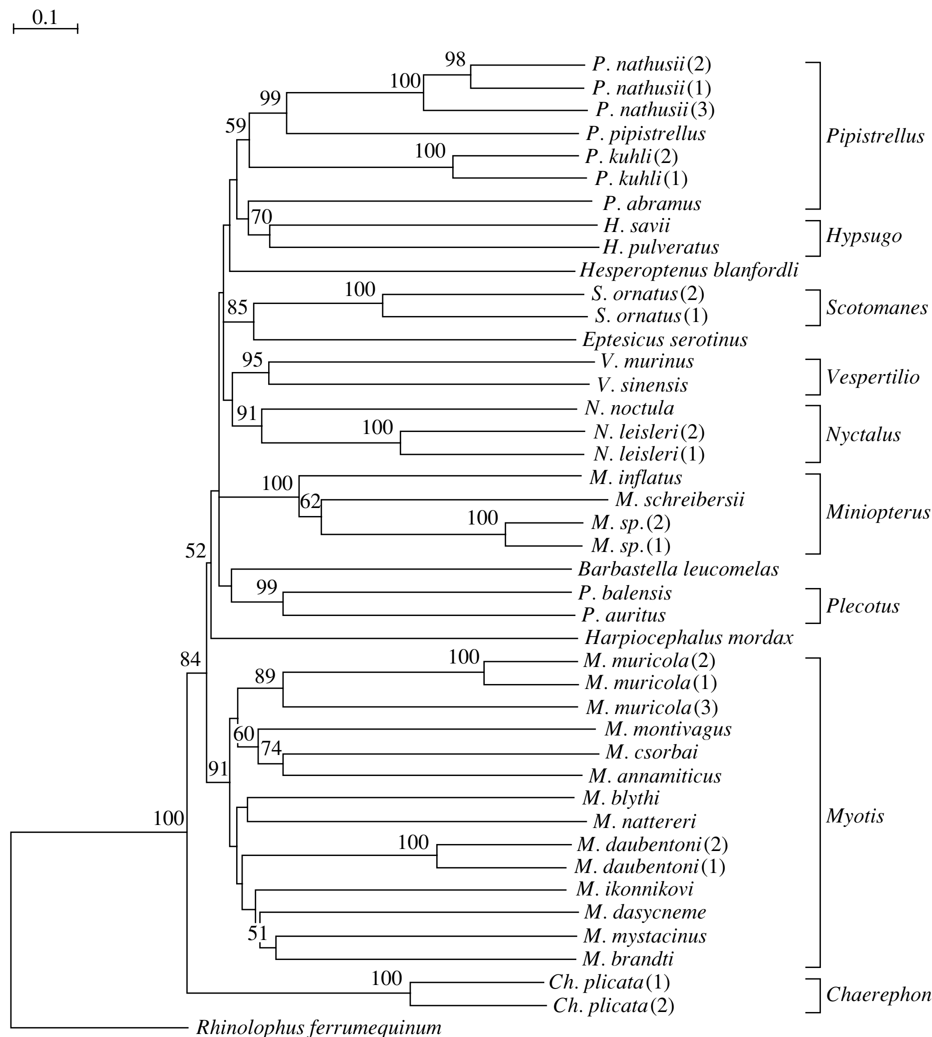

Fig. 5. NJ dendrogram of phylogenetic relationship in the bat subfamily Vespertilionidae based on the MIR–PCR results. The species *Rhinolophus ferrumequinum* was used as an outgroup. Bootstrap indices for clusters with a validity below 50% are not shown. Sample numbers are given in parentheses.

rank, for instance, all species of this genus or genera as such. As a result, a valid tree topology supported by moderate and high BI was obtained only at low taxonomic levels, whereas the topology at the level of genus and supragenus was unresolved and fan-shaped because of low BI (lower than 50%). As an outgroup, we used a horseshoe bat species *R. ferrumequinum* (Fig. 5). Within Vespertilionidae, only the isolated position of two large sister clusters, one corresponding to the genus *Myotis* (BI = 85%) and the other including all other Vespertilionidae (BI = 65%), was valid. The isolated position of *Myotis* agrees with karyological evidence according to which the tribe Myotini has the same rank as subfamilies. Within *Myotis*, the cluster, in which *M. csorbai* and *M. annamiticus* formed a sister group in relation to *M. montivagus*, was valid (with BIs of 74 and 81%, respectively).

Within the cluster including all other Vespertilionidae, the isolated groups, which never the less did not have a fixed position on the dendrogram, formed the clusters of genera: *Miniopteris* (BI = 100%), *Plecotus* (BI = 100), *Vespertilio* (BI = 94%), and *Nyctalus* (BI = 70%). The fact that *Scotomanes ornatus* and *Eptesicus serotinus* fell into the same cluster (BI = 80%) is noteworthy. The possibility of uniting these genera into one or at least including them into one tribe is indicated by karyological and morphological data [38, 39]. The fact that *Pipistrellus* species fell into different clusters is also interesting. One of these clusters includes three European *Pipistrellus* species, *P. pipistrellus–P. nathusii* (BI = 97%) + *P. kuhli* (BI = 61%) and the other consists of two Asian *Hypsugo* species and *P. abramus*: *H. pulveratus–H. savii* (BI = 69%) + *P. abramus* (52%). The *H. pulveratus–H. savii* pair was characterized by a genetic distance typical of phylogenetically distant species included in the same genus ($D_L = 0.82$). This agrees with the current idea on the taxonomic position of *H. pulveratus* [40], which include this species in the genus *Hypsugo.* As for European *Pipistrellus* species, clustering *P. pipistrellus* with *P. nathusii* and not with *P. kuhli* agrees with a conventional viewpoint on a closer relationship between the former two species [41]. In addition to these conclusions, the following are also taxonomically valuable: *Myotis* bats deserve the rank of a separate subfamily (Myotinae); Murininae and Miniopterinae do not deserve the rank of independent families; *M. muricola* samples from Nepal and Cambodia are related to different species.

Some Specific Features, Benefits, and Limitations of the Inter-SINE–PCR Method

Recently, the RAPD markers become widespread in molecular systematics. However, the RAPD fingerprints are not always reproducible [42], and intraspecific polymorphism pertaining to these markers often results in imperfect estimation of genetic distances between taxa of supraspecies rank [43]. A methodical advantage of inter-SINE–PCR over the RAPD analysis is a higher information capacity and better reproducibility of results. Because of using very short primers (9 to 11 bp in length) in RAPD–PCR, this method appears to be more sensitive to even small deviations from specified PCR conditions, which explains frequently observed poor reproducibility of results. Increasing the length of arbitrary primer in RAPD–PCR results in a decrease in the number of fragments detected. Therefore, to obtain reliable and informative results, one has to conduct many PCR trials with many short arbitrary primers, whereas a single PCR trial with only one primer is enough to obtain the same and even greater amount of fragments in inter-SINE–PCR.

For inter-SINE–PCR, one should know consensus sequence for at least one SINE family specific of the studied taxon. Compared to RAPD–PCR, this is a disadvantage of inter-SINE–PCR, although it can be overcome. First, in all mammals, MIR–PCR can be used thanks to the presence of MIR copies in all representatives of this class. In addition, for most mammalian orders, SINEs and their nucleotide sequences are already known ([6–17] and references therein). Second, universal methods for detection and cloning of SINEs have recently been developed [44], which enable one to establish nucleotide sequences of new SINEs within a relatively short time.

Using MIR–PCR for analyzing interspecific genomic polymorphism in Lipotyphla and Chiroptera we could obtain a significantly greater number of amplified fragments in the latter taxon under similar experimental conditions. This agrees with data of Jurka *et al.* [14], which indicate that Lipotyphla and Rodentia have a considerably lower number of MIR copies than other mammals. The authors think that such a reduced number of MIR copies can be explained by more intense degradation of MIR copies in these taxa. In our opinion, another likely explanation is that the rate of amplification of MIR element is dissimilar in different phyletic branches. Nevertheless, a significantly greater amount of bands on fingerprints in bats, compared to hedgehogs, does not promote better resolution of the obtained tree. This is due to a small number of shared fragments, which is reflected in grouping genera. The latter fact can be explained by presumably ancient divergence of genera and (or) especially by high mutation rate in bat DNA.

Thus, it should be admitted that the taxonomic level for which inter-SINE–PCR produces the most informative and reliable phylogenetic data can vary among different groups of organisms. Our results indicate that inter-SINE–PCR is a simple and reliable tool for studying both phylogeny (at the levels of the species and the genus) and intraspecific polymorphism.

ACKNOWLEDGMENTS

We are grateful to all who helped or directly participated in collecting zoological material for this study.

We especially thank R.I. Dzuev, V.N. Vasilenko, and other staff members of the Kabardino-Balkar State University for their invaluable help during our work in the Caucasus region; V.A. Dolgov, N.A. Formozov, and A.B. Vasil'eva (Moscow State University); and V.V. Grechko for helpful comments on this study.

This work was supported by the Russian Foundation for Basic Research, project nos. 00-04-49301 and 01-04-06493.

REFERENCES

1. Williams, G.K., Kubelik, A.R., Livak, J., *et al.*, DNA Polymorphisms Amplified by Arbitrary Primers Are Useful as Genetic Markers, *Nucleic Acids Res.*, 1990, vol. 18, no. 22, pp. 6531–6535.
2. Zietkiewicz, E., Rafalski, A., and Labuda, D., Genome Fingerprinting by Simple Sequence Report (SSR)-Anchored Polymerase Chain Reaction Amplification, *Genomics*, 1994, vol. 20, pp. 176–183.
3. Vos, P., Hogers, R., Bleeker, M., *et al.*, AFLP: A New Technique for DNA Fingerprinting, *Nucleic Acids Res.*, 1995, vol. 23, no. 21, pp. 4407–4414.
4. Kramerov, D.A., Kraev, A.S., Ryskov, A.P., and Skryabin, K.G., The Primary Structure of the Mouse Highly Repetitive Sequence Homologous to Double-Stranded Pre-mRNA Regions, *Dokl. Akad. Nauk SSSR,* 1980, vol. 252, pp. 241–244.
5. Singer, M.F., SINEs and LINEs: Highly Repeated Short and Long Interspersed Sequences in Mammalian Genomes, *Cell* (Cambridge, Mass.), 1982, vol. 28, pp. 433–434.
6. Okada, N., SINEs, *Curr. Opin. Genet. Dev.,* 1990, vol. 1, pp. 498–504.
7. Serdobova, I.M. and Kramerov, D.A., Short Retroposons of the B2 Superfamily: Evolution and Application for the Study of Rodent Phylogeny, *J. Mol. Evol.*, 1998, vol. 46, pp. 202–214.
8. Kramerov, D.A., Evidence for the Phylogenetic Relatedness of the Families Gliridae and Sciuridae Based on the Analysis of the B1-DID Short Retroposon, *Dokl. Akad. Nauk*, 1999, vol. 364, no. 2, pp. 277–280.
9. Shimamura, M., Yasue, H., Ohshima, K., *et al.*, Molecular Evidence from Retroposons That Whales Form a Clade within Even-Toed Ungulates, *Nature,* 1997, vol. 388, pp. 666–670.
10. Shedlock, A.M. and Okada, N., SINE Insertions: Powerful Tools for Molecular Systematics, *BioEssays,* 2000, vol. 22, no. 2, pp. 148–160.
11. Nelson, D.L., Interspersed Repetitive Sequence Polymerase Chain Reaction (IRS-PCR) for Generation of Human DNA Fragments from Complex Sources, *Methods, Companion Methods Enzymol.*, 1991, vol. 2, pp. 60–74.
12. Kaukinen, J. and Varvio, S.-L., Artiodactyl Retroposons: Association with Microsatellites and Use in SINEmorph Detection by PCR, *Nucleic Acids Res.*, 1992, vol. 20, pp. 2955–2958.
13. Smit, A. and Riggs, A., MIRs Are Classic tRNA-Derived SINEs That Amplified Before the Mammalian Radiation, *Nucleic Acids Res.*, 1995, vol. 23, no. 1, pp. 98–102.
14. Jurka, J., Zietkiewicz, E., and Labuda, D., Ubiquitous Mammalian-Wide Interspersed Repeats (MIRs) Are Molecular Fossils from the Mesozoic Era, *Nucleic Acids Res.,* 1995, vol. 23, no. 1, pp. 170–175.
15. Gilbert, N. and Labuda, D., Evolutionary Inventions and Continuity of CORE-SINEs in Mammals, *J. Mol. Biol.*, 2000, vol. 298, pp. 365–377.
16. Buntjer, J.B., *DNA Repeats in the Vertebrate Genome as Probes in Phylogeny and Species Identification*, Netherlands: Utrecht Univ., 1997, pp. 1–130.
17. Borodulina, O.R. and Kramerov, D.A., Short Interspersed Elements (SINEs) from Insectivores: Two Classes of Mammalian SINEs Distinguished by A-Rich Tail Structure, *Mamm. Genome*, 2001, vol. 12, pp. 779–786.
18. Madsen, O., Scally, M., Douady, C.J., *et al.*, Parallel Adaptive Radiations in Two Major Clades of Placental Mammals, *Nature,* 2001, vol. 409, pp. 610–614.
19. Killian, J.K., Buckley, T.R., Stewart, N., *et al.*, Marsupials and Eutherians Reunited: Genetic Evidence for the Theria, *Mamm. Genome*, 2001, vol. 12, no. 7, pp. 513–517.
20. Karrol, E., *Paleontologiya i evolyutsiya pozvonochnykh* (Vertebrate Paleology and Evolution), Moscow: Mir, 1993, vol. 3, pp. 1–312.
21. Arrigi, F.E., Bergendahl, G., and Mandel, M., Isolation and Characterization of DNA from Fixed Cells and Tissues, *Exp. Cell Res.*, 1968, vol. 50, pp. 47–53.
22. Maniatis, T., Fritsch, E.F., and Sambrook, J., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York: Cold Spring Harbor Lab., 1982.
23. Swofford, D.L., *PAUP*: Phylogenetic Analysis Using Parsimony (*And Other Methods), Ver. 4,* Sunderland, Mass.: Sinauer Associates, 1998.
24. Van de Peer, V. and De Wachter, R., TREECON: A Software Package for the Construction and Drawing of Evolutionary Trees, *Cabios*, 1994, vol. 9, no. 2, pp. 177–182.
25. Link, W., Dixens, C., Sirigh, M., *et al.*, Genetic Diversity in European Mediterranean Faba Bean Germ Plasm Revealed by RAPD Markers, *Theor. Appl. Genet.*, 1995, vol. 90, pp. 27–32.
26. Corbett, G.B. and Hill, J.E., *Mammals of the Indo-Malayan Region: Systematic Review*, Oxford Univ. Press, 1992.
27. Zaitsev, M.V., To the Systematics and Diagnostics of Hedgehog Species of the Subgenus *Erinaceus* (Mammalia, Erinaceidae) from the Fauna of the Soviet Union, *Zool. Zh.*, 1984, vol. 63, no. 5, pp. 720–729.
28. Filippucci, M.G. and Simson, S., Allozyme Variation and Divergence in Erinaceidae (Mammalia, Insectivora), *Israel J. Zool.*, 1996, vol. 42, pp. 335–345.
29. Santucci, F., Emerson, B.C., and Hewitt, G.M., Mitochondrial DNA Phylogeography of European Hedgehogs, *Mol. Ecol.*, 1998, vol. 7, pp. 1–10.
30. Bannikova, A.A., Fedorova, L.V., Fedorov, A.N., *et al.*, A Comparison of Repetitive DNA Sequences in Mammals of the Family Erinaceidae by Means of Restriction Enzyme Analysis, *Genetika* (Moscow), 1995, vol. 31, no. 11, pp. 1498–1506.

31. Sokolov, V.E., *Sistematika mlekopitayushchikh* (Mammalian Systematics), Moscow: Vysshaya Shkola, 1973.
32. Frost, D.R., Wozencraft, W.C., and Hoffmann, R.S., Phylogenetic Relationships of Hedgehogs and Gymnures (Mammalia: Insectivora; Erinaceidae), *Smithsonian Contribution to Zoology,* 1991, vol. 518, pp. 1–69.
33. Hutterer, R., Order Insectivora, *Mammal Species of the World,* Wilson, D.E. and Reeder, D.A., Eds., Smithsonian Inst., 1993, pp. 69–130.
34. Yates, T.L., Insectivores: Elephant Ahrews, Tree Shrews and Dermopterans, *Orders and Families of Recent Mammals of the World*, Anderson, S. and Knox, J., Eds., New York, 1984, pp. 117–144.
35. Anderson, S. and Jones, K., *Recent Mammals of the World,* New York: Ronald, 1967.
36. Robbins, C.B. and Setzer, H.W., Morphometrics and Distinctness of the Hedgehog Genera (Insectivora: Erinaceidae), *Proc. Biol. Soc.* (Washington), 1985, vol. 98, pp. 112–120.
37. Corbet, G.B., The Family Erinaceidae: A Synthesis of Its Taxonomy, Phylogeny, Ecology and Zoogeography, *Mamm. Rev.*, 1988, vol. 18, no. 3, pp. 117–172.
38. Volleth, M., Karyotyp Evolution and Phylogenie der Vespertilionidae (Mammalia: Chiroptera), *Den Naturwissenschaftlichen Fakultaten der Friedrich-Alexander-Universitat Erlangen-Nurnberg zur Erlangung des Doktorgrades*, 1989, pp. 1–262.
39. Volleth, M. and Heller, K.-G., Phylogenetic Relationships of Vespertilionid Genera (Mammalia: Chiroptera) as Revealed by Karyological Analysis, *Z. Zool. Syst. Evolut.-Forsch*, 1994, vol. 32, pp. 11–34.
40. Hill, J.E. and Harrison, D.L., The Baculum in the Vespertilioninae (Chiroptera: Vespertilionidae) with a Systematic Review, a Synopsis of *Pipistrellus* and *Eptesicus*, and the Description of a New Genus and Subgenus, *Bull. Br. Mus. Nat. Hist.*, 1987, vol. 52, pp. 225–305.
41. Horacek, I. and Hanak, V., Generic Status of *Pipistrellus savii* and Comments on Classification of the Genus *Pipistrellus* (Chiroptera, Vespertilionidae), *Myotis*, 1985–1986, vols. 23–24, pp. 9–16.
42. Backeljau, T., De Briun, L., Wolf, H., *et al.*, Random Amplified Polymorphic DNA (RAPD) and Parsimony Methods, *Cladistics*, 1995, vol. 11, pp. 119–130.
43. Rothuizen, J. and Van Wolveren, M., Randomly Amplified DNA Polymorphisms in Dogs Are Reproducible and Display Mendelian Transmission, *Anim. Genet.*, 1994, vol. 25, pp. 13–18.
44. Borodulina, O.R. and Kramerov, D.A., Wide Distribution of Short Interspersed Elements among Eukaryotic Genomes, *FEBS Lett.,* 1999, vol. 457, pp. 409–413.