Molecular Biology, Vol. 32, No. 6, 1998, pp. 840–845. Translated from Molekulyarnaya Biologiya, Vol. 32, No. 6, 1998, pp. 998–1003.
Original Russian Text Copyright © 1998 by Yurchenko, Martinkina, Merzlyak, Vengerov, Kolesnikov.

GENE MOLECULAR BIOLOGY

UDC 576.893.161.13

Characterization of *Trypanosoma scelopori* Kinetoplast DNA: Conserved Region of Minicircle as a Molecular-Taxonomic Feature

V. Yu. Yurchenko[1], L. P. Martinkina[2], E. M. Merzlyak[1], Yu. Yu. Vengerov[2], and A. A. Kolesnikov[1]

[1] *Faculty of Biology, Moscow State University, Moscow, 119899 Russia;*
E-mail: (A.A.K.) sasha@protein.bio.msu.su, (V.Yu.Yu.) slava@protein.bio.msu.su
[2] *Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, 117984 Russia*

Received April 12, 1998

Abstract—The minicircle kDNA component of *Trypanosoma scelopori* (a parasite of Iguanidae) was characterized. Three classes of minicircles differing in size were demonstrated. Analysis of the minicircle conserved region confirmed the close relationship of *T. scelopori* and *T. cruzi* postulated from other molecular parameters. The evolutionary relationships inside this group of Protozoa were analyzed using the DNA sequence of the minicircle conserved region as a genetic marker. The possibility of using this attribute for phylogenetic reconstructions is shown.

Key words: kinetoplast DNA, minicircle, conserved region, protists, phylogeny, *Trypanosoma scelopori*

INTRODUCTION

The origin and evolution of parasitism has long ago attracted the researchers' attention. Different groups of parasites have different directions of evolution. Flagellates possessing different variants of the life cycle and parasitizing in the circulation or alimentary tract form a small and compact group within which one can trace some evolutionary changes. In the framework of the most popular hypothesis, the monogenetic life cycle (one invertebrate host) should precede the digenetic life cycle (vertebrate and invertebrate hosts) [1]. Two opposite opinions on "the primary parasitic host" (vertebrate or invertebrate) were stated as regards the time and ways of digenetic cycle evolvement [2].

The hypothesis proposed by Minchin [3] and developed by Lavier [4] and Wallace [5] postulates that ancient trypanosomal parasitic systems colonized the gut and subsequently the blood of early aquatic vertebrates. By infecting Hirudinea and then hematophagous insects, they spread among various groups of both aquatic and terrestrial vertebrates. This hypothesis explains why trypanosomes are found in practically all classes of vertebrates and why the hematophagous insects are included in almost all parasitic systems.

The other hypothesis pioneered by Leger [6] and elaborated by Grasse [7], Baker [8], and Hoare [9] claims that the parasites of vertebrates originated from the parasites of hematophagous insects and leeches. According to this model, the parasitism arose in ancient invertebrates that got their parasites from a common annelid-like ancestor, and then evolved together with the hosts.

Both hypotheses postulate antiquity of the monogenetic life cycle of parasites. However, recent studies on Kinetoplastida phylogeny reconstruction using molecular approaches disagree with the general paradigm [1, 10]. Moreover, the earliest diverging branch is represented by two digenetic parasites of Mammalia—*Trypanosoma brucei* and *T. cruzi*, while the monogenetic parasites of insects form a more recently diverging group [11]. This evolutionary tree led to the conclusion that the ancestral trypanosomatids were actually digenetic [12] and the monogenetic parasites of insects have originated therefrom [13].

Analyzing the polyphyletic origin of digenetic parasites, Molyneux has assumed a possibility of simultaneous existence of digenetic parasites and monogenetic parasites of insects as sisterly or very early diverged lineages [14]. However, there is no close relation between the parasites of Mammalia—*T. brucei* and *T. cruzi*—on the one hand, and parasites of insects, on the other. This is quite explainable considering the early divergence of the mammalian parasites [15].

0026-8933/98/3206-0840$20.00 © 1998 МАИК Наука/Interperiodica Publishing

The sequence of minicircular conserved region, including the replication origins for both DNA strands, is a widely used genetic marker in phylogeny of trypanosomatids [16]. A variety of minicircles from different trypanosomal systematic groups analyzed to date allow tracing the evolutionary events within these groups [17].

The kDNA of trypanosomatids consists of catenated mini- (ca. $5 \cdot 10^3$ to $5 \cdot 10^4$ per associate) and maxicircular (20–50 per associate) molecules [17]. The genetic function of minicircles remained unclear until it was shown that the *Leishmania tarentolae* minicircles [18] encode guide RNA (gRNA) molecules involved in the RNA editing of maxicircular cryptogenes. Moreover, it turned out that the bulk of gRNAs is encoded in the minicircular component of kDNA.

All trypanosomal kDNA minicircles are organized by the same scheme and contain one or several conserved regions (CR) (up to 90%) and nonconserved regions. There are three highly conserved (90–100% homology) blocks (CSB) inside the CR: CSB1 (GGGCGT), CSB2 (CCCCGTTC), CSB3 (GGGGTTGGTGTA); CSB1 and CSB3 are practically identical in all species investigated to date, whereas CSB2 is less universal [19]. It was shown that CSB1 and CSB3 take part in the minicircle DNA replication initiation process.

We studied the evolutionary relations inside *Trypanosoma*, one of the most ancient genera in this parasitic group.

Unfortunately, the main part of lower vertebrate's trypanosomes have not been described until recently. Almost all of them were characterized only morphologically [16, 20, 21]. For example, we have scarce information about amphibian and reptilian trypanosomes; the primary restriction analysis of amphibian *T. mega* minicircles was carried out in 1977 [22], and for reptilian *T. platydactyli* in 1988[20], but the structural and functional features of these minicircles remain unclear.

The aim of this study was to analyze the minicircular component of the lizard trypanosome (*T. scelopori*) kDNA and to assess the evolutionary relationships between the parasites of animals from various taxonomic groups, using the sequence of minicircular CR as a genetic marker.

EXPERIMENTAL

The strain of *T. scelopori* [23], kindly provided by Dr. J. Lukesh (Institute of Parasitology, Ceske Budejovice, CR), was isolated from a desert lizard *Sceloporus jarrovi* (Sceloporinae, Iguanidae) captured in 1995 in Arizona (USA). The flagellates were introduced into modified SNB-9 medium and cultivated at 26°C [24].

Cells from stationary phase culture ($5 \cdot 10^7$ cells/ml) were pelleted at 5000 rpm, washed with an equal volume of the NET-50 buffer (50 mm EDTA, 100 mm NaCl, 10 mm Tris, pH 8.0) and lysed for 1 h on ice with 3% N-sarcosyl (Fluka) and 1mg/ml pronase E (Merck). The lysate was centrifuged for 110 min at 115,000 *g* in a Beckman SW 28Ti rotor. A pellet of kDNA was extracted twice by phenol-chloroform and centrifuged for 45 min at 130,000 *g* in a Beckman SW 60Ti rotor. Pellets were rinsed with ethanol, dried, and resuspended in 10 mM TE buffer [16]. The total yield of isolated kDNA was 0.5–1 μg per 10^7 cells.

The restriction endonucleases and T4 DNA ligase were from Promega; the QIAquick GelExtraction kit, QIAquick PCR purification kit, and QIAprep spin miniprep kit were from QIAGEN.

The vector pUC19 (Fermentas, Lithuania) and *E. coli* XL1Blue (Stratagene) were used for molecular cloning of PCR-amplified fragments. *Hin*dIII and *Bam*HI sites were added to the ends of the primers.

The PCR was run in a Perkin-Elmer 9600 PCR machine: 94°C/3 min; then 30 cycles: 94°C/30 s, 55°C/1 min, 72°C/1 min 30 s; extension 10 min at 72°C. The structures of primers were:

CSB1: 5'-TTGGATCCAGTTGCACGCCCGTCCCGA-3'
CSB3: 5'-GTTAAGCTTGGTGCTTCGATAGGGGTTGG-3'
CSB3in: 5'-TTGGATCCTATCGAAGCACCAC-3'
CSB3out: 5'-TTAAGCTTGGGGTTGGTGTA(G/A)AAA-3'

Sequencing was performed using a Perkin-Elmer–Cetus automatic device.

Electron microscopy of the samples was done according to Davis *et al.* [25].

The following software packages were used in analysis: Dnasis (Hitachi Software Engineering Co., Ltd), GeneBee, PCGENE-6.5 (IntelliGenetics Inc. and Genofit SA 1991), DNASTAR for Windows (ver.3.03, 3.04 1993–1995), NCBI-BLAST. The phylogenetic analysis was carried out using GeneBee and TREECON [26] software packages. The analyzed minicircular DNAs were from freshwater fish trypanosome *T. carassii* (GenBank acc. no. S82304) and mammalian trypanosomatids *T. congolense* (M19750),

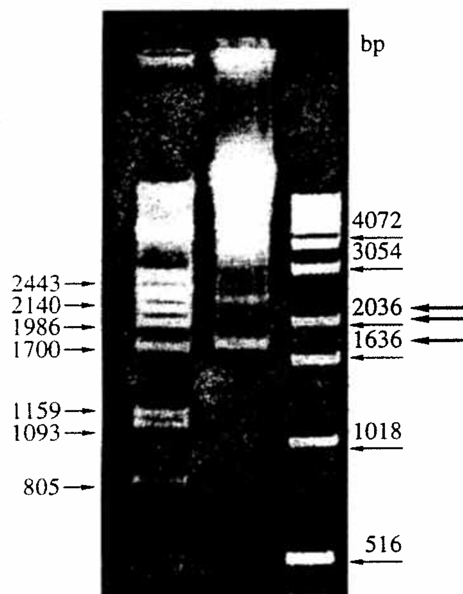

Fig. 1. The characteristic of *Trypanosoma scelopori* kDNA minicircles. Electrophoretic separation of *T. scelopori* kDNA in 1% agarose gel (1× TAE buffer). The arrows mark three classes of minicircles. The sizes of fragments are in base pairs.

Fig. 2. Electron microscopy of *Trypanosoma scelopori* kDNA. The molecule of the major class (ca. 1700 bp in size) is presented. The scale bar is 0.1 μm.

T. lewisi (M17995), *T. brucei* (J01454, V01388), *T. cruzi* (U07845). The sequence of *Trypanoplasma borrelii* (U14185) CR was used as an outgroup.

RESULTS AND DISCUSSION

The Minicircular Component of *T. scelopori* kDNA

Agarose gel separation of *T. scelopori* kDNA revealed at least three minicircular classes with the sizes of 1700 (major), 2050, and 2300 bp (minor) (Fig. 1). The amount of the minor classes kDNA was less than 1/15 of the total minicircle DNA (Fig. 1).

Cleavage of the total minicircle pool with a restriction enzyme which has a 4-bp recognition site (*Msp*I, *Taq*I) is a common method for estimating molecular heterogeneity [20]. Analysis of the *T. scelopori* kDNA associate digested with *Msp*I testified to low heterogeneity for basic class molecules (data not shown).

The organization features of the *T. scelopori* kDNA associate were revealed by electron microscopy. The associate had the morphology normal for all trypanosomes investigated to date, however, it exhibited a quite high extent of compaction. The pool of minicircle molecules was represented by both compacted and decompacted molecular forms. This allowed us to estimate the correct size of the minicircle. Figure 2 shows the molecule of the basic class (1700 bp).

For primary assessment and analysis of the *T. scelopori* minicircle organization, use was made of PCR with primers annealing in the CSB1 and CSB3 regions. These sites are practically identical among the trypanosomes studied (Fig. 3, primers CSB1 and CSB3). The variable regions between neighboring CSB1 and CSB3 were amplified (Fig. 3). No frames for the known genes or gRNA genes (as shown for *T. cruzi* [27]) were found in the analyzed sequence. From these data (GenBank acc. nos. AF044842–AF044844) we chose the correct primer pair for analysis of the complete minicircle CR (Fig. 3, primers CSB3in and CSB3out). The PCR product of ca. 420 bp (AF044840, AF044841) contained the full-length CR 5'-flanked by fragments of variable regions (Fig. 3). The appearance of the 420-bp PCR product on the 1700-bp template demonstrates that minicircles from the *T. scelopori* associate are multimeric (e.g., four CRs are almost symmetrical within one minicircle). The multimeric organization was found for a number of *Trypanosoma* species (for example, *T. cruzi* has four CRs in the major minicircular class [28] and *T. carassii* has two CRs [29]).

The *T. scelopori* CRs (191 bp) are arranged in the same way as all investigated trypanosomal CRs. They contain conserved blocks CSB1, CSB2, and CSB3, and also a DNA bend in the 5' direction from CSB3 (Fig. 4). The sequences of CSB 1 and CSB3 are identical to all investigated CRs, and the less conserved sequence CSB2 (CCCCGTAC) differs from the most frequently occurring sequence (CCCCGTTC) by one nucleotide (T ⟶ A). The sequence of the bend does not differ from the consensus. This fragment may play an important role in the initiation of minicircle replication [17], but this is obviously an auxiliary mechanism, because the minicircles without a bend are also maintained in natural selection [29]).

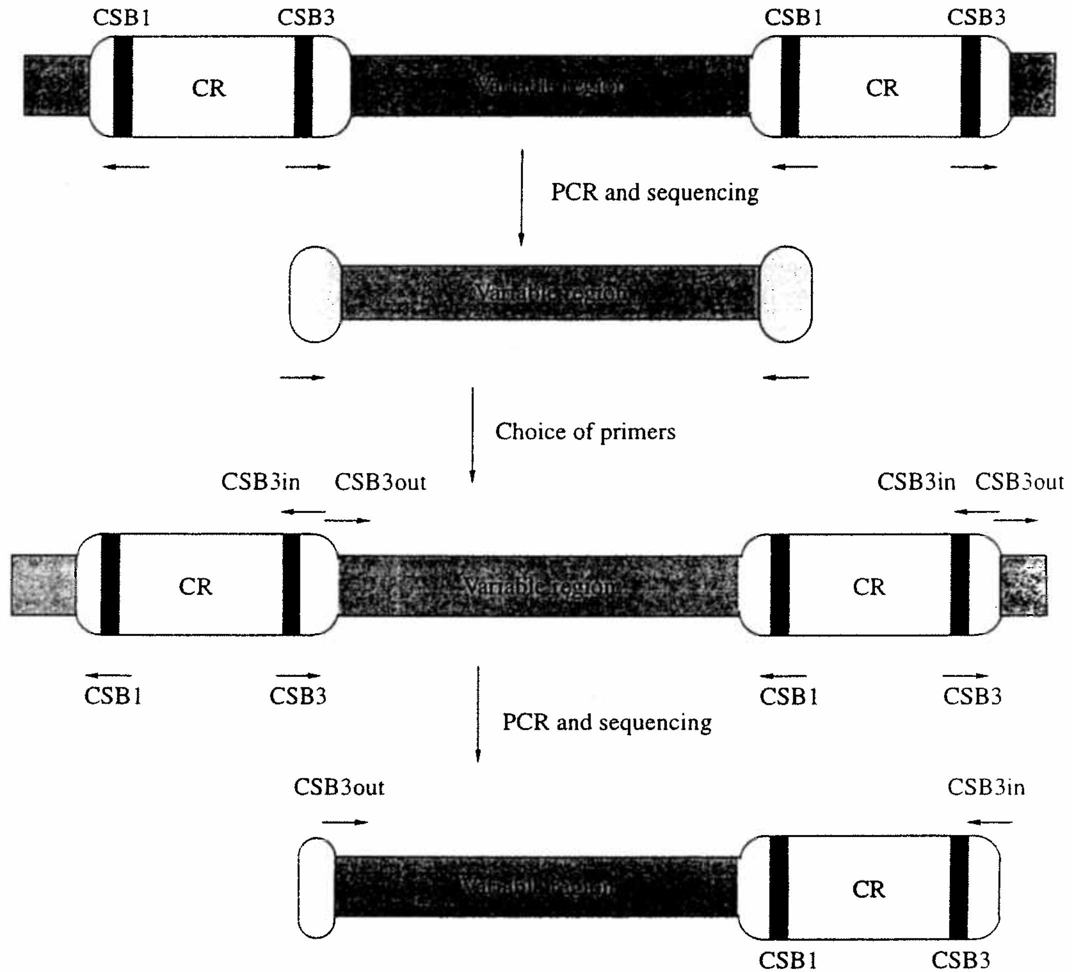

Fig. 3. The experimental scheme. The analysis of minicircular variable regions by PCR with primers CSB1 and CSB3 (GenBank acc. nos. AF044842–AF044844) has allowed to pick out the primers from the 3' fragments of CR (primers CSB3in and CSB3out). The complete minicircular CR was amplified and analyzed using these primers (AF044840, AF044841).

Use of Minicircle CR Sequence as Taxonomic Marker

There are several molecular markers allowing reconstruction of the evolutionary relations inside the group of parasitic protozoa.

(i) **Nuclear large and small subunit rRNA genes** [1, 13, 30, 31]. These are the classical markers for molecular phylogenetic reconstructions. They are the most neutral with respect to mutation accumulation and hence to selection. Therefore these markers are frequently used as reference for subsequent trees [2].

(ii) **Kinetoplast 9S and 12S rRNA genes** [30, 32]. The phylogenetic tree of a large number of trypanosomal genera built using these parameters showed early divergence of *T. brucei* from the basic Trypanosomatidae evolution lineage, confirming the antiquity of the *Trypanosoma* genus.

(iii) **Edited mitochondrial genes** [30]. The character and degree of editing (distribution of U in preedited mRNA molecules) allows detection of the subsequent evolutionary events [11].

In this study, the phylogenetic tree of minicircle CRs was built using the Jukes–Cantor algorithm.

The fragments between CSB1 and CSB3 with flanking regions (222 bp) were used in the analysis [16]. Our preliminary attempts to use the fragment of 104 bp between CSB1 and CSB3 as such for building the phylogenetic tree proved unsuccessful. Such trees were "unstable," e.g., adding any new sequence changed the tree dramatically (data not shown). We concluded that the 104-bp fragment of CR cannot be

TTGAAATAATTTATGTTAAACTTTATTAAAAATCCAGTTCAAAAAAATTT 50

CSB1

CCGGGAGCCCAAAATTTAGGGGGCGTTCAACTTTATAGCCCAAAAATAG 100

CSB2

CGCATGTCCCCCGTACAAAAAATGGCCCAAAAACGCTAATTTTCACGGA 150

CSB3

GTGGTGCTTCGATAGGGGTTGGTGTAGAAATATTTATGTT 191

Fig. 4. The sequence of *Trypanosoma scelopori* minicircle CR. The CSB1, CSB2, and CSB3 conserved blocks are underlined. The DNA sites participating in the bend formation are framed.

Crithidia fasciculata
Trypanosoma carassii var. esox
Trypanosoma carassii var. abramis
Trypanosoma carassii
Trypanosoma cruzi
Trypanosoma scelopori
Trypanosoma congolence
Trypanosoma lewisi
Trypanosoma brucei
Trypanoplasma borelli

Fig. 5. The phylogenetic tree showing the relations inside the Trypanosomatidae family, constructed by the TREECON program using the Jukes–Cantor algorithm.

used for evolutionary studies. In further analysis we considered only fragments of 222 bp.

The phylogenetic tree constructed on the CR nucleotide sequences is presented in Fig. 5. This tree coincides with the trees constructed using other molecular markers, first of all (i) above [10, 13, 30]:

(a) It supports the hypothesis [31] about the early divergence of *T. brucei* from the basic evolution lineage.

(b) The divergence of the African (*T. lewisi, T. congolense*) group lineage from the group of freshwater fish trypanosomes is apparent.

(c) *T. scelopori* and *T. cruzi* form a monophyletic group [10], which may be indicative of the common ways of their minicircular kDNA evolution. This is quite interesting because these species are transmissed by different vectors (Diptera or Hemiptera); *T. cruzi* parasitizes in the blood of Mammalia (warm-blooded animals) and *T. scelopori* in the blood of Reptilia (cold-blooded animals).

In brief, the sequence of minicircular CR can be used as a simple molecular marker. The phylogenetic tree constructed with this marker favors the view that the digenetic life cycle of Trypanosomatidae was actually the primary one.

ACKNOWLEDGMENTS

The authors thank K. Alexandrov and A. Iakovenko (Max-Planck Institute for Molecular Physiology, Dortmund, Germany) for their technical help and assistance at all steps of the study; A. V. Troitsky (Belozersky Institute of Physico-Chemical Biology, Moscow) for the program TREECON and his help in phylogenetic tree construction; J. Lukesh, M. Jirku, and D. Dolezel (Institute of Parasitology, Ceske Budejovice, CR) for the strain of *T. scelopori* and helpful discussion of the paper. This work was supported by the Russian Foundation for Basic Research (project nos. 96-04-49186, 98-04-49037, 98-04-49247).

REFERENCES

1. Maslov, D.A., Lukes, J., Jirku, M., and Simpson, L., *Mol. Biochem. Parasitol.*, 1996, vol. 75, pp. 197–205.

2. Lainson, R. and Shaw, J.J., *The Leishmaniases in Biology and Medicine*, Peters, W. and Killick-Kendrick, R., Eds., London: Acad. Press, 1987, pp. 1–120.
3. Minchin, E.A., *Q. J. Microsc. Sci.*, 1908, vol. 52, pp. 159–260.
4. Lavier, G., *Annu. Parasitol.*, 1943, vol. 19, pp. 168–196.
5. Wallace, F.G., *Exp. Parasitol.*, 1966, vol. 18, pp. 124–193.
6. Leger, L., *Comp. Rend. Seances Soc. Biol. Ses Fil.*, 1904, vol. 56, pp. 615–617.
7. Grasse, P.-P., *Traite de Zoologie*, Grasse, P.-P., Ed., Paris: Masson, 1952, pp. 603–668.
8. Baker, J., *Exp. Parasitol.*, 1963. vol. 13, pp. 219–233.
9. Hoare, C.A., *The Trypanosomes of Mammals*, Oxford: Blackwell Scientific Publications, 1972.
10. Lukes, J., Jirku, M., Dolezel, D., Kral'ova, I., Hollar, L., and Maslov, D.A., *J. Mol. Evol.*. 1997, vol. 44, pp. 521–527.
11. Maslov, D.A., Avila, H.A., Lake, J.A., and Simpson, L., *Nature*, 1994, vol. 365, pp. 345–348.
12. Landweber, L.F. and Gilbert, W., *Proc. Natl. Acad. Sci. USA*, 1994, vol. 91, pp. 918–921.
13. Fernandes, A.P., Nelson, K., and Beverley, S.M., *Proc. Natl. Acad. Sci. USA*, 1993, vol. 90, pp. 11608–11612.
14. Molyneux, D.H., *Leishmania. Taxonomie et Phylogenese. Applications eco-epidemiologiques*, Montpellier: IMEEE, 1984, pp. 231–240.
15. Woo, P.T.K., *Nature*, 1970, vol. 228, pp. 1059–1062.
16. Jirku, M., Kolesnikov, A.A., Benada, O., and Lukes, J., *Mol. Biochem. Parasitol.*, 1995. vol. 73, pp. 279–283.
17. Simpson, L., *Int. Rev. Cytol.*, 1986, vol. 99, pp. 119–179.
18. Sheline, C. and Ray, D.S., *Mol. Biochem. Parasitol.*, 1989, vol. 37, pp. 151–157.
19. Ray, D.S., *Mol. Cell. Biol.*, 1989, vol. 9, pp. 1365–1367.
20. Simpson, L. and Holz, G., Jr., *Parasitology*, 1988, vol. 4, pp. 115–118.
21. Lopez, T. and Melton, C.G., Jr., *J. Parasitol.*, 1975, vol. 61, pp. 209–212.
22. Borst, P., Fase-Fowler, F., Steinert, M., and van Assel, S., *Exp. Cell. Res.*, 1977, vol. 110, pp. 167–173.
23. Ayala, S.G., *J. Protozool.*, 1970, vol. 17, pp. 370–373.
24. Diamond, L.S. and Herman, C.M., *J. Parasitol.*, 1954, vol. 40, pp. 195–202.
25. Davis, R.W., Simon, M., and Davidson, N., *Methods Enzymol.*, McCormick, D.W. and Wright, L.D., Eds, London: Acad. Press, 1971, vol. 21D, pp. 413–427.
26. Van de Peer, Y. and De Wachter, R., *Comput. Applic. Biosci.*, 1994, vol. 10, pp. 569–570.
27. Avila, H.A. and Simpson, L., *RNA*, 1995, vol. 1, pp. 939–947.
28. Frasch, A.C.C., Sanchez, D.O., and Stoppani, A.O.M., *Biochim. Biophys. Acta*, 1984, vol. 782, pp. 26–33.
29. Baron, L.I., Yurchenko, V.Yu., and Kolesnikov, A.A., *Mol. Biol.*, 1995, vol. 29, pp. 1341–1348.
30. *RNA Editing: The Alternation of Protein Coding Sequences of RNA*, Benne, R., Ed., Hemel Hempstead, Herts, UK: Simon and Schuster, 1993.
31. Gomez, E., Valdes, A.M., Pinero, D., and Hernandez, R., *Mol. Biol. Evol.*, 1991, vol. 8, pp. 254–259.
32. Du, Y., Maslov, D.A., and Chang, K.-P., *Proc. Natl. Acad. Sci. USA*, 1994, vol. 91, pp. 8437–8441.