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PHYLOGENETIC RELATIONSHIPS BETWEEN THE SIX SUPEROXIDE DISMUTASE PROTEINS (FESOD) OF *TRICHOMONAS VAGINALIS* AND FESOD6 GENETIC DIVERSITY

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Summary :

The parasitic protozoan *Trichomonas vaginalis* is known to contain several types of Fe-containing superoxide dismutase proteins (FeSOD). Using three different methods of phylogenetic analysis, maximum parsimony (MP), neighbor joining (NJ), and maximum likelihood (ML) methods, we examined the phylogenetic relationships among the six FeSOD (FeSOD1-FeSOD6) based on their amino acid sequences. All the analyses consistently suggested that the six proteins formed a monophyletic group implying that they probably be originated from an ancestral protein form through repeated duplication events. Although MP tree was totally unresolved, the NJ and ML trees revealed that FeSOD6 placed the most basal position and thus emerged earlier than the other five gene types during the evolution of *T. vaginalis*. Phylogenetic relationships among the five remaining proteins were (FeSOD2, FeSOD3), (FeSOD4, (FeSOD1, FeSOD5)) although weakly supported in terms of bootstrapping values. In addition to this, we newly designed two PCR primer specifically amplifying full-length FeSOD6 gene and examined its genetic diversity among 12 *T. vaginalis* isolates from five countries and three continents. They had the same nucleotide sequences except those of three Korean isolates which showed one to three different nucleotides

KEY WORDS : *Trichomonas vaginalis*, FeSOD, molecular phylogeny, FeSOD6 genetic diversity.

Résumé : RELATIONS PHYLOGÉNÉTIQUES ENTRE LES SIX PROTÉINES SUPEROXIDE DISMUTASE (FESOD) DE *TRICHOMONAS VAGINALIS* ET LA DIVERSITÉ GÉNÉTIQUE DE FESOD6

Trichomonas vaginalis *est un protozoaire parasite qui possède plusieurs protéines superoxide dismutase riches en fer (FeSOD). Les relations phylogénétiques entre les six protéines (FeSOD1-FeSOD6) ont été examinées en utilisant les méthodes d'analyse phylogénétique suivantes : parcimonie, distance et maximum de vraisemblance. Toutes les analyses ont montré que les six protéines formaient un groupe monophylétique issu vraisemblablement d'une protéine ancestrale par des événements de duplication. Bien que l'arbre représentatif de l'analyse de parcimonie montre une irrésolution entre les six protéines, les arbres de distance et de maximum de vraisemblance placent la protéine FeSOD6 en position ancestrale. Les relations phylogénétiques entre les cinq autres protéines ne sont pas toutes bien défendues en terme de valeurs de bootstrap (robustesse aux nœuds), elles se présentent sous la forme (FeSOD2, FeSOD3), (FeSOD4, (FeSOD1, FeSOD5)). Deux nouvelles amorces, ont été définies pour amplifier l'intégralité du gène FeSOD6, ce qui a permis d'étudier sa diversité génétique à partir de 12 isolats de* T. vaginalis *en provenance de cinq pays et de trois continents. Il s'est avéré que toutes les séquences étaient identiques, à l'exception de trois isolats coréens qui présentaient une à trois bases de différence.*

MOTS CLÉS : Trichomonas vaginalis, *FeSOD, phylogénie moléculaire, diversité génétique de FeSOD6.*

INTRODUCTION

Trichomonas vaginalis, a common cause of vaginitis and exocervicitis, is a microaerophilic protozoan parasite that possesses many characteristics of anaerobic organisms, including sensitivity to oxygen. According to a recent report (World Health Organization, 1995), the annual incidence of trichomoniasis exceeds 170 million cases worldwide. Adequate defense against oxidative stress usually requires the presence of a number of protective mechanisms. *T. vaginalis* consumes oxygen at low levels and oxygen consumption systems are present in the cytosome and in the hydrogenosomes (Ellis *et al.*, 1994). Superoxide dismutase (SOD) converts superoxide anions (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) which, in turn, are metabolized by catalase and peroxidase (Bannister *et al.*, 1987). Three different SOD classes, namely those of FeSOD (prokaryotes, plants, and protozoa), MnSOD (prokaryotes, mitochondria, and chloroplasts), and Cu/ZnSOD (higher animals, and plants) have been published to date. They differ in their metal contents and in terms of their sensitivities to cyanide, azide, and hydrogen peroxide (Michelson *et al.*, 1977; Tannich *et al.*, 1991). It is known that *T. vaginalis* has at least 7 FeSOD gene types, among which five gene types (from FeSOD1 to FeSOD5) have been partially characterized, but only the FeSOD6 gene type has been completely characterized (Viscogliosi *et al.*, 1996, 1998). Viscogliosi *et al.* (1996) examined the phylogenetic relationships among

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FeSOD1-FeSOD5 types by the neighbor joining (NJ) method on the basis of partial amino acid sequences, but did not study the phylogenetic position of FeSOD6. In addition, no one has researched the genetic diversity of the FeSOD types among *T. vaginalis* isolates in parallel with geographical distribution studies.

In this study, we reconstructed the phylogenetic relationships of the six FeSOD types to elucidate the phylogenetic position of the FeSOD6, and examined the degree of genetic diversity of the FeSOD6 type in the 12 *T. vaginalis* isolates collected from five countries on three different continents. Newly designed FeSOD6 gene-specific PCR primers were used to specifically amplify full-length FeSOD6 gene.

MATERIALS AND METHODS

Sample collection and cellular DNA extraction

Twelve *T. vaginalis* isolates were obtained from five geographically distinct regions (six Korean, one Japanese, one Chinese, one Australian, and three American) (Table I). *T. vaginalis* isolates were grown axenically at 37° C in TYM (Trypticase-Yeast extract-Maltose) medium and total genomic DNA was extracted from *T. vaginalis* isolates using the phenol-chloroform extraction method (Brindley *et al.,* 1993).

Phylogenetic analysis

To examine phylogentic relationship among the six FeSOD types of *T. vaginalis*, partial amino acid sequences from the six FeSOD types of *T. vaginalis* (accession no. Z70670-Z70674 and AF022423), *T. gallinarium* (Z70676), *Monocercomonas* sp. (CAA94524), *Hypotrichomonas acosta* (CAA94522) and *Tritrichomonas foetus* (AAC47734) were retrieved from EMBL data bank. Except for the six from *T. vaginalis*, the remaining ones were used as reference groups for phylogenetic analysis, of which *T. foetus* was employed as an outgroup. Amino acid sequence alignment of FeSOD from *T. vaginalis* and other five trichomonads (Fig. 1) and nucleotide sequence alignment of FeSOD6 genes from twelve *T. vaginalis* isolates (Fig. 3) were constructed by the Clustal X multiple alignment program (Thompson *et al.*, 1997) for phylogenetic analysis and sequence comparison. Phylogenetic analysis was conducted using maximum parsimony (MP) and neighbor joining (NJ) methods of PAUP* 4.0 beta 8 (Swofford, 2000) and maximum likelihood (ML) method of Puzzle 4.0 (Strimmer & von Haeseler, 1996). In MP and NJ analyses, bootstrapping analysis with 1,000 replicates was performed to estimate the degree of confidence of each clade. In ML, quartet puzzling (1,000 steps) was conducted for the same purpose.

Strains	Geographic origin
NYH286	ATCC 50148
CDC85	America (Ohio)
RU393	ATCC 50142
IR78	Austria (Vienna)
YA1	Japan (Chiba)
KT19	China (Yonbyun)
KT4	Korea (Seoul)
KT11	Korea (Kuri)
KT12	Korea (Kuri)
KT18	Korea (Wonju)
KT38	Korea (Song-Nam)
KT40	Korea (Song-Nam)

Table I. – *Trichomonas vaginalis* isolates used in this study.

Full-length PCR amplification of FeSOD6 gene and DNA sequencing

The entire FeSOD6 gene was amplified by PCR using *Taq* DNA polymerase (Promega, Madison, Wisconsin). We used two newly designed primers, SODFL-1 (5'-ATG TTC ACA ATG GAG CAT CCT GCC-3', 5'-end position 1-24) and SODFL-2 (5'-TTA CAA ACC AGC AGC CTT-3', 3'-end position 568-585), to specifically amplify the complete SOD coding region of the FeSOD6 gene type from *T. vaginalis* isolates, by referring to the report of Viscogliosi *et al.* (1998). PCR was carried out using the following conditions: 94° C for five min (one cycle); and 30 cycles of 94° C for one min, 60° C for one min, and 72° C for one min; and finally 72 for 10 min (one cycle). PCR products were purified by extraction from agarose gel using QIAXII (Qiagen, Chatsworth, California). Purified PCR products were cloned into the pGEM T-easy vector system following the manufacturer instructions (Promega). The ligation mixture was used to transform $CaCl_2$-competent *E. coli* TOP10. White colonies were selected by the X-gal/IPTG method and plasmid DNA was isolated using the previously published alkaline method (Sambrook *et al.*, 1989). DNA sequencing was performed with an Li-COR automatic DNA sequencer (model 4200) using T7 forward and SP6 reverse primers.

RESULTS AND DISCUSSION

Monophyletic origin of *T. vaginalis* FeSOD and phylogenetic position of FeSOD6

The amino acid sequences of six FeSOD protein types of *T. vaginalis* and FeSOD proteins from five trichomonad reference taxa were aligned as shown in Figure 1, which was used for the present phylogenetic analyses with MP, NJ, and ML methods

Taxon	Sequence	Position
T. vaginalis 1	QAYIDTANKLIVGSGFEGKSIEEVIQKAQGPLFNNVAQHFNHSFFWKCLSADKVAVPAKV	[60]
T. vaginalis 2	...............L.................................TPE..D..S..	[60]
T. vaginalis 3	...............L.................................TPE..D..S..	[60]
T. vaginalis 4	...............L...................................E........	[60]
T. vaginalis 5	...............L...................................E........	[60]
T. vaginalis 6	.S.................P...I.......................S...E......H.	[60]
T. gallinarum	...............L...TM..I..A.S..........Y..Q...R..TPK..EI....	[60]
H. acosta	R..V.M....VPE.PLN......I..NST..I.......Y..A...NS.T.Q.QEI.PS.	[60]
M. sp.	...V.MT...VP.TE....T..DIVKTSS..I...I...Y......D..T.Q.QDI..P.	[60]
T. foetus	...V.F...NVP.TE....P...I....T.............A...N..T.K.QE...G.	[60]
T. vaginalis 1	ADALTKEFGSVEKFEETFTAKASTVFGSGWCYLYKNKEGKLEIGQYSNAANPVKDGFKPV	[120]
T. vaginalis 2	..V.ASN.E.....K.......................S.....................	[120]
T. vaginalis 3	..V.ASN.E.....K......................D..C...............H..L	[120]
T. vaginalis 4	..............Q......................D..C...............H..L	[120]
T. vaginalis 5	..............Q.......................S.................H...	[120]
T. vaginalis 6	.EL.K.N.......Q...............A....T.D..................LT.I	[120]
T. gallinarum	KAV.E.........K.A.QG....L.....A....S.........F...L......GV.I	[120]
H. acosta	.AF.I.H.K..DD.KKD.V..............AA.QD.SIS.N...........HGF.I	[120]
M. sp.	QEF...H.Q..D..KTD.V..............AQ.ADKSIS.N...........NGF.I	[120]
T. foetus	.SF.A.H.E..DN.KAQ.VQ.............AQ..DKTIS.N.....L......GV.L	[120]
T. vaginalis 1	LTVDT	[125]
T. vaginalis 2	.....	[125]
T. vaginalis 3	.....	[125]
T. vaginalis 4	.....	[125]
T. vaginalis 5	.....	[125]
T. vaginalis 6	.....	[125]
T. gallinarum	.A...	[125]
H. acosta	.....	[125]
M. sp.	.....	[125]
T. foetus	.C...	[125]

Fig. 1. – Amino acid sequence alignment of partial FeSOD1 ~ 6 of *Trichomonas vaginalis* and partial FeSOD from five reference taxa. This alignment was used for the phylogenetic analyses. *T. vaginalis* 1 ~ 6, partial FeSOD1 ~ 6 of *Trichomonas vaginalis*; *T. gallinarum*, FeSOD of *Tetratrichomonas gallinarum*; *T. foetus*, FeSOD of *Tritrichomonas foetus*; *H. acosta*, FeSOD of *Hypotrichomona acosta*; *M.* sp., FeSOD of *Monocercomonas* sp. Dots indicate amino acids identical with those of the first line (FeSOD1 of *T. vaginalis*).

(Fig. 2). As a result, *T. vaginalis* FeSOD proteins consistently formed a monophyletic group with 74 %, 98 %, and 100 % bootstrap values in the MP, NJ, and ML analyses, respectively (Fig. 2). Although relationships among six FeSOD types were unresolved in the MP tree (Fig. 2A) except a sister relation of FeSOD2 and FeSOD3 (91 % bootstrapping value), the other two analyses, NJ and ML (Figs. 2B and 2C), showed that FeSOD6 is placed at the most basal position. It implied that FeSOD6 split off earlier than the others during the evolution of *T. vaginalis*. In the NJ and ML analyses, phylogenetic relationships among six FeSOD types were (FeSOD6, (FeSOD4,(FeSOD1, FeSOD5), (FeSOD2, FeSOD3))), but all the nodes were not supported by high bootstrapping values (Fig. 2) and the MP tree was unresolved. Notwithstanding these week results, the relationships among FeSOD1 ~5 types were the same as those obtained by Viscogliosi *et al.* (1996) (using NJ method and partial FeSOD1-FeSOD5 sequences), but different in that the closest FeSOD type of FeSOD5 was found to be FeSOD1 and not FeSOD4 but the bootstrapping value was relatively low in NJ (50 %) and ML analyses (52 %).

SPECIFICITY OF SODFL-1 AND -2 PRIMERS FOR AMPLIFICATION OF FESOD6 AND LOW GENETIC DIVERSITY OF THE FESOD6 GENE

The complete nucleotide sequences of FeSOD6 from 12 *T. vaginalis* isolates collected from five geographically distinct regions (six Korean, one Japanese, one Chinese, one Australian, and three American isolates) were amplified by PCR using SODFL-1 and -2 primers (see Materials and Methods) which are specific to FeSOD6 gene type. The obtained PCR products were cloned and sequenced. All the twelve FeSOD6 genes have the same lengths (585 bp) without any indels. The nucleotide sequence alignment of the FeSOD6 genes

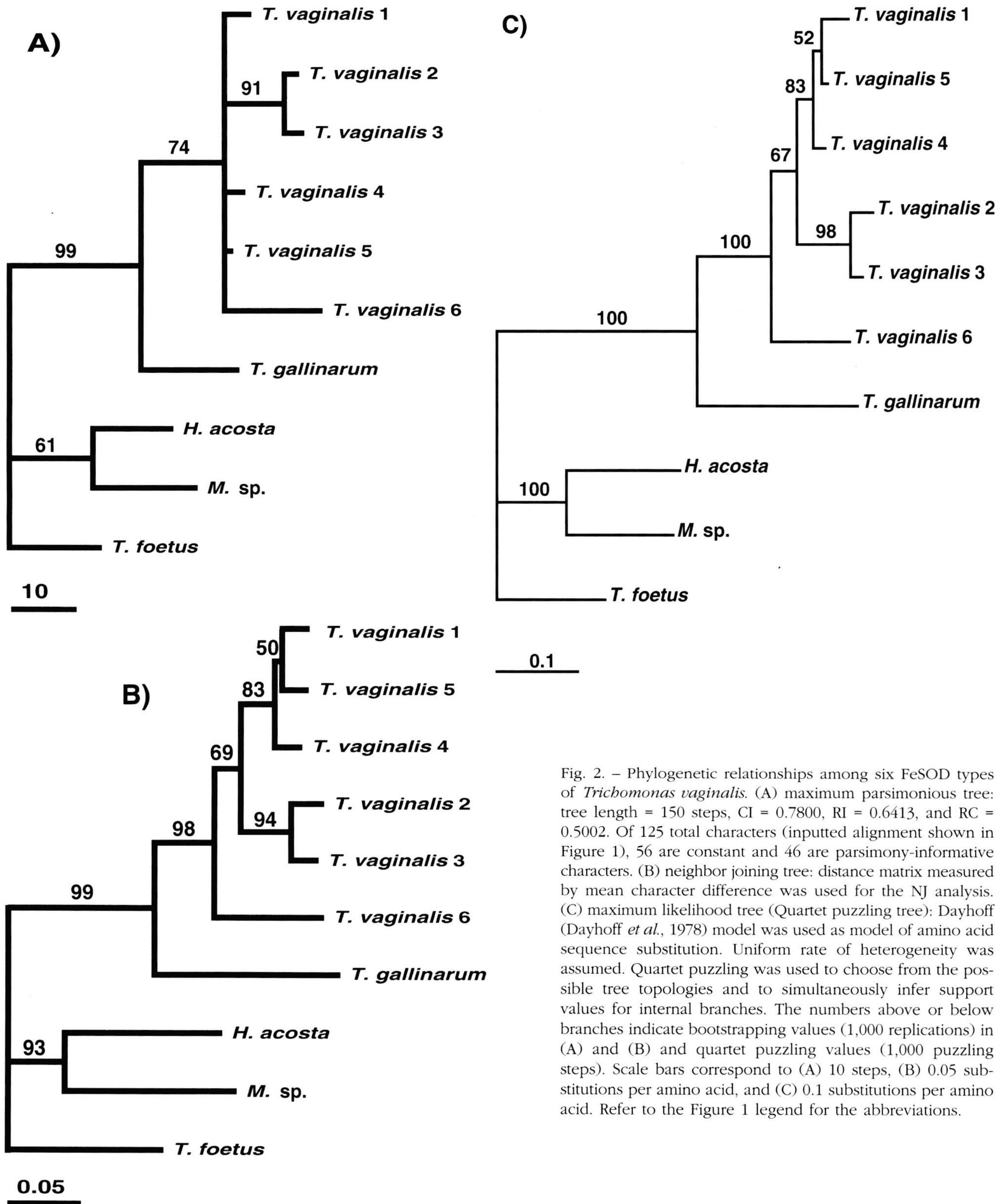

Fig. 2. – Phylogenetic relationships among six FeSOD types of *Trichomonas vaginalis*. (A) maximum parsimonious tree: tree length = 150 steps, CI = 0.7800, RI = 0.6413, and RC = 0.5002. Of 125 total characters (inputted alignment shown in Figure 1), 56 are constant and 46 are parsimony-informative characters. (B) neighbor joining tree: distance matrix measured by mean character difference was used for the NJ analysis. (C) maximum likelihood tree (Quartet puzzling tree): Dayhoff (Dayhoff *et al.*, 1978) model was used as model of amino acid sequence substitution. Uniform rate of heterogeneity was assumed. Quartet puzzling was used to choose from the possible tree topologies and to simultaneously infer support values for internal branches. The numbers above or below branches indicate bootstrapping values (1,000 replications) in (A) and (B) and quartet puzzling values (1,000 puzzling steps). Scale bars correspond to (A) 10 steps, (B) 0.05 substitutions per amino acid, and (C) 0.1 substitutions per amino acid. Refer to the Figure 1 legend for the abbreviations.

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TvSOD6*   ATGTTCACAATGGAGCATCCTGCCTACTTGAAGACTGGTCTTCCAGGCTTCCTCACACAG   [60]
KT4       ............................................................   [60]
KT12      ........................................................G...   [60]
KT18      ............................................................   [60]

TvSOD6*   CACGCTGTCGAGGTCCATGTTACAAAGCACCATCAGTCCTACATTGATACAGCTAACAAG   [120]
KT4       ............................................................   [120]
KT12      ....TC......................................................   [120]
KT18      ............................................................   [120]

TvSOD6*   CTTATCGTTGGCTCTGGCTTCGAAGGCAAGCCAATTGAAGAAATCATCCAAAAGGCTCAG   [180]
KT4       ............................................................   [180]
KT12      ............................................................   [180]
KT18      ............................................................   [180]

TvSOD6*   GGCCCACTCTTCAACAACGTTGCCCAGCACTTCAACCACTCCTTCTTCTGGAAGTCCCTC   [240]
KT4       ............................................................   [240]
KT12      ............................................................   [240]
KT18      ............................................................   [240]

TvSOD6*   TCCGCTGAGAAGGTTGCTGTTCCAGCTCATGTTGCTGAGCTCCTCAAGAAGAACTTCGGC   [300]
KT4       ............................................................   [300]
KT12      ............................................................   [300]
KT18      ............................................................   [300]

TvSOD6*   TCTGTCGAGAAGTTCCAGGAAACATTCACAGCTAAGGCTTCAACAGTCTTCGGCTCTGGC   [360]
KT4       ............................................................   [360]
KT12      ............................................................   [360]
KT18      .....T......................................................   [360]

TvSOD6*   TGGGCTTACCTTTACAAGACAAAGGACGGCAAGCTTGAGATCGGCCAGTACTCCAACGCT   [420]
KT4       .............................T..............................   [420]
KT12      ............................................................   [420]
KT18      .....................................G......................   [420]

TvSOD6*   GCTAACCCAGTCAAGGATGGCCTTACACCAATTCTCACAGTCGATACATGGGAACATGCT   [480]
KT4       ............................................................   [480]
KT12      ............................................................   [480]
KT18      ............................................................   [480]

TvSOD6*   TGGTACATCGACTACGAGAACAGAAAGGCTGAGTACTTCAAGAACTACTGGAACCACGTC   [540]
KT4       ............................................................   [540]
KT12      ............................................................   [540]
KT18      ............................................................   [540]

TvSOD6*   AACTGGAACTTTGTTGAGCAGAACTTAAAGGCTGCTGGTTTGTAAA   [586]
KT4       ..............................................   [586]
KT12      ..............................................   [586]
KT18      ..............................................   [586]
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Fig. 3. – Nucleotide sequence alignment of complete FeSOD6 genes from 12 *Trichomonas vaginalis* isolates collected from five geographically distinct regions (six Korean, one Japanese, one Chinese, one Australian, and three American isolates) with previously reported one (TvSOD6). * indicates that FeSOD6 gene sequences of the nine isolates (NYH286, CDC85, RU393, IR78, YA1, KT19, KT11, KT38, and KT40) are identical with that of the previously reported TvSOD6 gene (AF022423). Dots indicate nucleotide identical with those of the first line (TvSOD6). Refer to Table I for additional information of isolates.

from the twelve isolates with a previously reported FeSOD6 gene (accession no. AF022423) is shown in Figure 3. The obtained sequence comparison showed that genetic differences of *T. vaginalis* FeSOD6 gene from the different geographical areas do not exist with the exception of a small number of nucleotide changes in the three Korean isolates, KT4 (1 bp), KT12 (3 bp), and KT18 (2 bp). This result also showed that the new primer set designed in the present study is highly specific to the FeSOD6 gene type.

CONCLUSION

Phylogenetic analysis was performed with six FeSOD types revealed that all are monophyletic and FeSOD6 emerged earlier than the other types during the evolution of *T. vaginalis*. It suggests that the six *T. vaginalis* FeSOD proteins could be originated from an ancestral protein form through repeated duplication events. FeSOD6 gene sequence comparison of *T. vaginalis* isolates from various countries and continents showed that the genetic diversity of FeSOD6 is very poor, since none difference appeared between the isolates from different countries and continents and curiously the very small differences (one to three different nucleotides) were observed in three isolates from a same geographic origin. In addition, two newly designed PCR primers are highly specific to FeSOD6 gene.

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