BIOLOGIA PLANTARUM 56 (1): 140-144, 2012

BRIEF COMMUNICATION

Molecular cloning and characterization of nucleoside diphosphate kinase 1 cDNA in tea

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Abstract

Nucleoside diphosphate kinase (NDPK) operates in the homeostasis of cellular nucleoside triphosphate (NTP) pools and the cytosolic NDPK1 is the main NDPK isoform in plants, accounting for more than 70 % of total NDPK activity in plant. A full length cDNA (697 bp), designated as *CsNDPK1* was cloned from *Camellia sinensis* (L.) O. Kuntze leaves. Sequence analysis of *CsNDPK1* shows several motifs, binding and catalytic sites which are highly conserved among other NDPKs. Southern blot analysis revealed that tea genome has two copies of *CsNDPK1*. Transcription pattern analysis indicated that *CsNDPK1* is expressed in all tissues examined, but expressed more in buds than in other organs.

Additional key words: *Camellia sinensis,* cytosolic expression, nucleoside triphosphate pool, sequence analysis, Southern blot.

Nucleoside diphosphate kinases (NDPKs, EC 2.7.4.6) are ubiquitous housekeeping enzymes that catalyze the ping-pong mechanism of γ-phosphate group transfer from 5′-triphosphonucleotides (NTPs) to 5′-diphosphonucleosides (NDPs). In plants, they are widely distributed and extensively characterized (Johansson *et al*. 2008). Three different isoforms of NDPK are distinguished: NDPK1, which lacks identifiable targeting sequence and it is localized in the cytosol, NDPK 2 and NDPK3, which have N-terminal organellar targeting signals and are therefore localized in plastids or mitochondria. While NDPK2 and NDPK3 have been characterized in details (Janin *et al*. 2000, Larsen, 2001, Hasunuma *et al*. 2003, Dorion *et al*. 2006), characterization of NDPK1 is still very scarce (Nomura *et al*. 1992, Pan *et al*. 2000). Further characterization of NDPK isoforms in plants will improve our understanding on their diverse roles in plant biology. In this study, we report the cloning, sequence and expression characterization of NDPK1 gene (*CsNDPK1*) from tea.

Tea is one of the most popular beverages in the world owing to its taste, stimulative effect, and health benefits. Approximately 562 full-length characterized genes and/or proteins from tea plant have been reported so far (http://www.ncbi.nlm.nih.gov/protein?term=camellia%20sinensis). Most of them are important enzymes related to secondary metabolism, and stress or disease resistance (Chunlei and Liang 2007). To our knowledge, this is the first report of cloning and characterization of a full-length *NDPK1* gene in tea. In addition, semi-quantitative RT-PCR analysis was performed to determine the expression pattern of this gene in tea tissues.

Tea [*Camellia sinensis* (L.) O. Kuntze] clone UPASI-3 (Assam type; Balasaravanan *et al*. 2003), growing well in the tea garden of the UPASI- Tea Research Institute (latitude 10º 30´N, longitude 27º 0'S and altitude 1 050 m a.s.l.) was selected for RNA and DNA isolation.

Total RNA and DNA were isolated from the tissue samples using the RNeasy plant mini kit and plant DNeasy mini kit (*Qiagen,* USA) as per the manufacturer's instructions, respectively. Single stranded cDNA of transcripts were prepared in 25 mm^3

Received 23 March 2010, *accepted* 19 October 2010.
Abbreviations: DTT - dithiothreitol, EST - expressed sequence tag; NDPK - nucleoside diphosphate kinase; NTP - nucleoside triphosphate; PCR - polymerase chain reaction; RACE - rapid amplification of cDNA ends.
Acknowledgements: Authors are thankful to Dr. P. Mohan Kumar, Director and Dr. N. Muraleedharan, Adviser of UPASI Tea Research Foundation for their encouragement and support during the course of study. Financial assistance from NTRF, Tea Board, Kolkata is also gratefully acknowledged.
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reaction volume containing 5× first strand reverse transcription buffer, 10 mM dithiothreitol (DTT), 50 µM dNTP, 200 U of *Power-script* reverse transcriptase (*Clontech*, USA), 2.0 µg of total RNA and 50 pmol *Oligo* $T_{(15)}$ for 60 min at 42 ºC and a final denaturation step at 72 ºC for 7 min. The standard RACE PCR protocol was carried out using expressed sequence tag (EST) specific primers (P1: 5′-ATGGAGCGGACTTTCGTCA-3′ for 3′RACE and P2: 5′-TTCATTCATAGATCCACAGAG TGAAG-3′ for 5′RACE) obtained in our earlier study and universal primers provided in the *SMART*™ RACE cDNA amplification kit (*Clontech*) as per the manufacturer's instructions. The PCR was carried out in an *Mastercycler* gradient PCR machine (*Eppendorf*, Germany) under the following conditions: 95 °C (4 min), 27 cycles of 95 °C (30 s), 63 °C (1 min), and 68 °C (2 min). The PCR products were analyzed on 1 % agarose gel, purified using *QIAquick* gel extraction kit (*Qiagen*), and cloned into T/A cloning vector *pTZ57R/T* (*Fermentas*, USA). The ligated products were transformed into *E. coli* strain DH5α and propagated on ampicillin (100 µg cm^{-3}) supplemented Luria broth (LB)

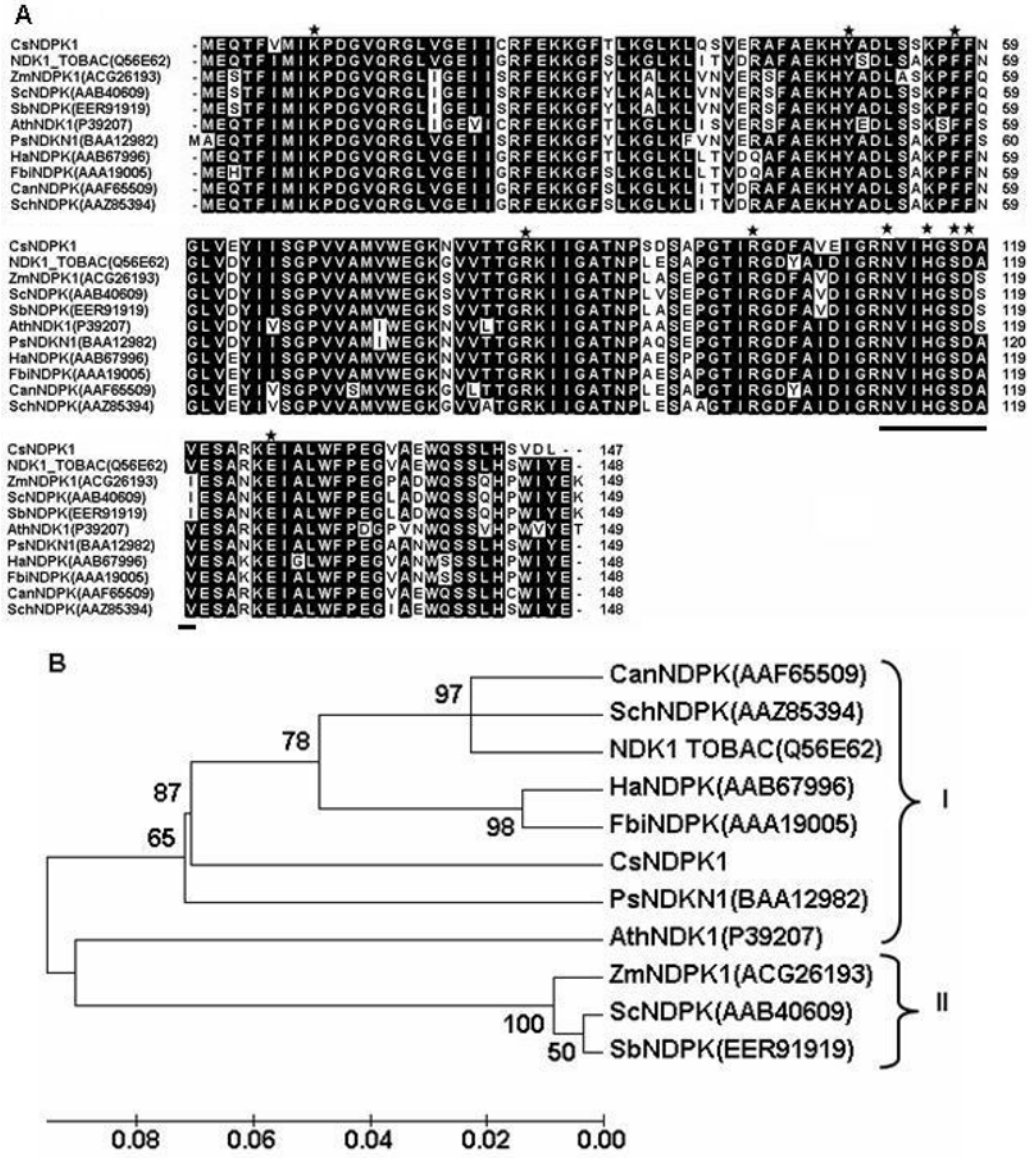

Fig. 1. *A* - Comparison of the deduced amino acid sequence of CsNDPK1 with other NDPKs from various organisms. The accession Nos. of the sequences used in the comparison are provided in the parentheses, tobacco NDK1 (Q56E62), capsicum NDK (AAF65509), *Solanum* (AAZ85394), *Helianthus* (AAB67996), pea NDK1 (BAA12982), *Flaveria* NDPK (AAA19005), *Arabidopsis* NDK1 (P39207), maize (ACG26193), sorghum (EER91919) and sugarcane (AAB40609), and indicated in the left margin. Conserved active site residues are marked with *asterisks*. The NDP kinase active site motif (N-x-x-H-[G/A]-S-D-[SA]-[LIVMPKNE]) is underlined. Sequence data was deposited in the *GenBank* (Acc. No. GU332636). *B* - Un-rooted dendrogram derived from the amino acid sequences. The tree was generated from a consensus of 1000 bootstrap replicates using Neighbor-Joining method in *MEGA 4* program. The *scale bar* indicates the relative amount of change along branches.

agar plates. Plasmids isolated from selected clones were sequenced in an automated DNA sequencer (*ABI PRISM 3730XL* analyzer (*Applied Biosystems*, USA) using the *BigDye* terminator cycle sequencing kit and the sequencing primers M13F and M13R from both the ends of the insert. The nucleotide sequence data obtained were entered into the *BLAST* query and searched for sequence identity and similarity against the *NCBI* database (Altschul *et al*. 1997).

The RACE PCR protocol gave amplification in the 5' as well as 3'RACE reactions. The 5'RACE PCR produced a 546 bp product and the 3'RACE product was approximately 601 bp. *BLAST* analysis revealed the identity of the sequence of PCR products as nucleotide diphosphate kinase I isoform. Based on the RACE sequence data, the primers GP1 (5´-CTCGCTTGC CCATTTTCTCACTC-3´) and GP2 (5´-AAGATGCAG ATAAAACGTCGTGCA-3´) were designed and used to amplify the gene in full-length as a single PCR product under the following conditions: 94 ºC for 5 min, and 35 cycles of amplification of 94 ºC for 45 s, 61 ºC for 45 s, 72 ºC for 2 min and final extension of 72 ºC for 5 min. A single distinct product (697 bp) was obtained which was further cloned into T/A vector *pTZ57R/T* for sequence characterization using *BLAST* search as above. *BLAST* analysis identified the product as nucleoside diphosphate kinase I homologues, designated as *CsNDPK1*. Nucleotide sequence of *CsNDPK1* was submitted to *GenBank* (Acc. No. GU332636). The full-length *CsNDPK1* gene comprises 697 bp with 448 bp open reading frame (ORF), which encodes a deduced polypeptide of 147 amino acids, with a 96 bp long 5´UTR (untranslated regions) and a 153 bp long 3´UTR. The theoretical molecular mass (Mr) and isoelectric point (pI) of the deduced CsNDPK1 protein were predicted using *Protparam* (http://expasy.org/tools/protparam.html) to be 16.1 kDa and 6.3, respectively.

Further, the alignment and phylogenetic tree analysis of tea *CsNDPK1* nucleotide and protein with other structurally related NDPK genes were performed using the *ClustalW* and Neighbor-Joining method in *MEGA 4* program (Tamura *et al*. 2007). Multiple alignment analysis (Fig. 1*A*) by *Clustal W* indicated that *CsNDPK1* is sharing 89 % identity with tobacco NDK1 and 83 % with ATP binding NDPK1 of *Arabidopsis thaliana*. In addition, the consensus sequence (Gly-Xaa-Gly-Xaa-Xaa-Gly) present in various protein kinases and nucleotide binding proteins was also identified in CsNDPK1 (Gly^{12}-Val^{13}-Gln^{14}-Arg^{15}-Gly^{16}-Leu^{17}-Val^{18}-Gly^{19}; Hanks *et al*. 1988). The presence of Ile^{18} in this consensus sequence of monocot specific NDPKs is replaced with Val^{18} in dicot specific NDPKs. Similar dicot specific pattern is also observed in CsNDPK1 and thus, grouped into the subgroup I in phylogenetic tree, which includes the members of dicots (Fig. 1*B*).

In addition, *GenBank CDD* (conserved domain database) search and function analysis (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi) revealed that CsNDPK1 belongs to the nucleoside diphosphate kinase group I (NDPK I) family, which is characterized by a kinase conserved domain and conserved active site residues (Fig. 1*A*). The active site is highly conserved and the phosphor-histidine intermediate, identified by Gilles *et al*. (1991) corresponds to His^{115} in our deduced CsNDPK1 sequence (Fig. 1*A*). The observations are in accordance with the earlier studies in *Spinacia oleracia*, *Avena sativa*, *Pisum sativum* (Hasunuma *et al*. 2003).

There is no organelle targeting peptide in N-terminal region, thus CsNDPK1 is cytoplasmic protein as predicted by *iPSORT* prediction tool (http://hc.ims.u-tokyo.ac.jp/iPSORT/predict.cgi). In plants, 70 % of NDPK1 is found in the cytosol (Dorion *et al*. 2006), whereas NDPK2 and NDPK3 have N-terminal extensions targeting organelles (Gilles *et al*. 1991, Sweetlove *et al*. 2001, Dorion *et al*. 2006).

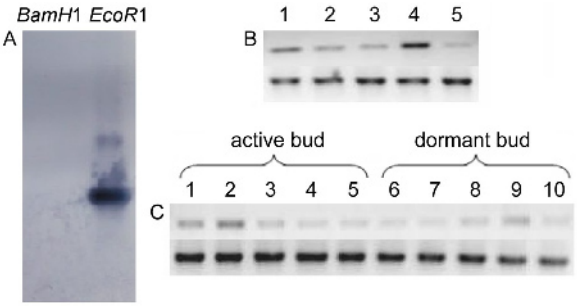

Fig. 2. *A* - Southern blot analysis of *CsNDPK1* copy number in tea genome. Expression analysis of *CsNDPK1* by semi-quantitative RT-PCR from different tissues (*B*; *lanes* 1 - bud, 2 - first leaf, 3 - second leaf, 4 - first internode, 5 - second internode) and from active and dormant bud tissues collected at different DAS (*C*; *lanes* 1 and 6 - 12th DAS, 2 and 7 - 24th DAS, 3 and 8 - 36th DAS, 4 and 9 - 48th DAS, 5 and 10 - 60th DAS).

Table 1. Distribution and expression ratio of *CsNDPK1* in different parts of tea shoot. The results are expressed as mean values of two-independent reactions ± SD.

Plant part	ER [%]	DAS	ER [%] active bud	dormant bud
Bud	51.21±1.13	12	59.16±0.47	18.36±0.68
1st leaf	24.88±0.41	24	109.20±2.14	17.50±0.28
2nd leaf	13.36±0.90	36	26.94±0.54	34.66±0.86
1st internode	80.92±5.66	48	17.80±0.37	93.08±1.57
2nd internode	4.08±0.05	60	43.53±0.17	42.02±1.50

The hydrophobicity or hydrophilicity scales and the secondary structure conformational parameters of CsNDPK1 were computed using *SOPMA* (Geourjon and Deleage 1995). *SOPMA* and *Hydropathy* plot analysis revealed that CsNDPK1 was predominantly α-helical globular protein, which mainly consisted of α-helices (48.30 %) and random coils (26.53 %), while sheet (17.01 %) and β-turn (8.16 %) as observed for tobacco and *Arabidopsis* NDPK1.

Southern hybridization was carried out with full

length cDNA of *CsNDPK1* as probe labeled with digoxigenin labeled dUTP (DIG-dUTP). *Bam*H1 and *Eco*RI digested genomic DNA (20 µg), separated electrophoretically in a 0.8 % (m/v) agarose gel (16 h, 20 V), was transferred to a positively charged *Hybond-N*+® membrane (*GE Healthcare*, USA) by capillary and fixed by incubation at 80 °C for 2 h as described by Sambrook *et al*. (1989) and in *DIG High Prime* DNA labeling and detection *Starter Kit I* (*Roche,* Germany). The hybridization of membrane with *CsNDPK1* probe was performed in *DIG Easy Hyb* buffer for 16 h at 42 °C. After hybridization, the membrane was stringently washed 2 × 5 min with the following buffers: 2× SSC + 0.1 % SDS at 42 °C, 1× SSC + 0.1 % SDS at 42 °C, 0.5× SSC + 0.1 % SDS at 65 °C and 0.1× SSC + 0.1 % SDS at 65 °C. The signals were then detected with *anti-DIG* antibody (*Roche*) as per the manufacturer's instructions. The analysis showed two bands in *Eco*R1 digested sample (Fig. 2*A*) indicating that *CsNDPK1* exists as two copies in tea genome. In addition, variants of *CsNDPK1* were not amplified during PCR using genomic DNA of tea as template with the same *CsNDPK1* specific primers (data not shown). This result confirms the two copy number nature of *CsNDPK1* rather than paralog gene in tea genome.

For analyzing the distribution and expression pattern of *CsNDPK1* transcript in different regions of tea shoot, semi-quantitative reverse transcription-PCR (qRT-PCR) was performed using the isolated total RNA collected from the tissue samples (bud, first leaf, second leaf, first, and second internodes). Similarly, the isolated total RNA of bud tissues from the active and dormant shoots collected at 12, 24, 36, 48 and 60 d after shear harvesting (DAS; between 09:00 and 10:00) were used for determining the *CsNDPK1* expression pattern variations during the different developmental stages of tea bud. For semi-QRT-PCR, 1 µg of isolated total RNA was used for single strand cDNA synthesis using first strand cDNA synthesis kit (*Clontech*). Synthesized cDNAs were diluted 10× and used as template in RT-PCR reaction using *CsNDPK1* gene specific primer (P1 and P2) and tea 26S rRNA (FP: 5′-CACAATGATAGGAAGAGCC GAC-3′; RP: 5′-CAAGGGAACGGGCTTGGCAGA ATC-3′) as internal control with following PCR conditions: 24 cycles of 94 ºC for 45 s, 60 ºC for 45 s, and 72 ºC for 1 min and final extension of 5 min at 72 ºC.

The intensity of bands was quantified using *ImageJ* software (http://rsb.info.nih.gov/ ij/) and normalized against Cs26S rRNA band intensity. The average expression ratio (ER) was calculated between the normalized relative intensity of target gene in test tissue sample and the control from two independent RT-PCR reactions. The results showed that *CsNDPK1* expressed higher in first internode (ER of 80.96 %) and bud (ER of 51.21 %) than in the fully expanded leaf (Fig. 2*B*, Table 1). NDPKs are expressed ubiquitously but in a spatiotemporal manner, and increased at specific stages of life in several organisms and in various types of cells under certain circumstances (Ishikawa *et al*. 2003). Primary cell wall formation in bud tissue cells and wall material deposition continues with the maturation of the internodal tissue and resulted in the observed high expression pattern of *CsNDPK1* in those tissues. These data provides for the first time direct evidence on role of NDPK1 activity in early growth of tea similarly as in other plants (Dorion *et al*. 2006).

Expression pattern of *CsNDPK1* in active and dormant bud tissues indicates its increasing expression in active bud up to 24 DAS, whereas the expression level remained constant during the remaining experimental period (Fig. 2*C*, Table 1). In contrast, the expression level is low in dormant bud during the dormancy period (up to 24 DAS) and then showed progressive increase during the conversion of dormancy to active growth phase (36 and 48 DAS) followed by comparable expression with active bud at 60 DAS (Table 1). *CsNDPK1* may play an important role in bud dormancy regulation (our unpublished data); therefore, distinct expression pattern of *CsNDPK1* should be taken into account when evaluating the impact of bud dormancy and their control mechanisms in tea.

In conclusion, the present study provides the first information of NDPK1 gene sequence, its conserved structural details and role in bud growth in tea. Further identification of proteins interacting with CsNDPK1 may help to unravel a potential role of this protein in intracellular communication and its physiological significance in tea.

References

Altschul, S.F, Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman D.J.: *Gapped BLAST* and *PSI-BLAST*: a new generation of protein database search programs. - Nucl. Acids Res. **25**: 3389-3402, 1997.

Balasaravanan, T., Pius, P.K., Rajkumar, R., Muraleedharan, N., Shasany, A.K.: Genetic diversity among South Indian tea germplasm (*Camellia sinensis*, *C. assamica* and *C. assamica* spp. *lasiocalyx*) using AFLP markers. - Plant Sci. **165**: 365-372, 2003.

Chunlei, M.A., Liang, C.: Research progress on isolation and cloning of functional genes in tea plants. - Front. Agr. China **1**: 449-455, 2007.

Dorion, S., Matton, D.P., Rivoal, J.: Characterization of a cytosolic nucleoside diphosphate kinase associated with cell division and growth in potato. - Planta. **224**: 108-124, 2006.

Geourjon, C., Deleage, G.: *SOPMA*: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. - Comput. Appl. Biosci. **11**: 681-684, 1995.

Gilles, A.M., Presecan, E., Vonica, A., Lascu, I.: Nucleoside diphosphate kinase from human erythrocytes. Structural characterization of the two polypeptide chains responsible for heterogeneity of the hexameric enzyme. - J. biol. Chem. **266**: 8784-8789, 1991.

Hasunuma, K., Yabe, N., Yoshida, Y., Ogura, Y., Hamada, T.: Putative functions of nucleoside diphosphate kinase in plants and fungi. - J. Bioenerg. Biomem. **35**: 57-65, 2003.

Hanks, S.K., Quinn, A.M., Hunter, T.: The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. - Science **241**: 42-52, 1988.

Ishikawa, N., Shimada, N., Takagi, Y., Ishijima, Y., Fukuda, M., Kimura, N.: Molecular evolution of nucleoside diphosphate kinase genes: conserved core structures and multiple-layered regulatory regions. - J. Bioenerg. Biomembr. **35**: 7-18, 2003.

Janin, J., Dumas, C., Morera, S., Xu, Y., Meyer, P., Chiadmi, M., Cherfils, J.: Three-dimensional structure of nucleoside diphosphate kinase. - J. Bioenerg. Biomembr. **32**: 215-225, 2000.

Johansson, M., Hammargren, J., Uppsall, E., Mackenzie, A., Knorpp, C.: The activities of nucleoside diphosphate kinase and adenylate kinase are influenced by their interaction. - Plant Sci. **174**: 192-199, 2008.

Larsen, K.: Molecular cloning and analysis of a cDNA coding for nucleoside diphosphate kinase from ryegrass. - J. Plant. Physiol. **158**: 267-272, 2001.

Nomura, T., Yatsunami, K., Honda, A., Sugimoto, Y., Fukui, T., Zhang, J., Yamamoto, J., Ichikawa, A.: The amino acid sequence of nucleoside diphosphate kinase I from spinach leaves, as deduced from the cDNA sequence.- Arch. Biochem. Biophys. **297**: 42-45, 1992.

Pan, L., Kawai, M., Yano, A., Uchimiya, H.: Nucleoside diphosphate kinase required for coleoptile elongation in rice. - Plant Physiol. **122**: 447-452, 2000.

Sambrook, J., Fritsch, E.F., Maniatis, T.H.R.S.G.: Molecular Cloning - A Laboratory Manual. 2nd Ed. - Cold Spring Habour Laboratory Press, New York 1989.

Sweetlove, L.J., Mowday, B., Hebestreit, H.F., Leaver, C.J., Millar, A.H.: Nucleoside diphosphate kinase III is localized to the inter-membrane space in plant mitochondria. - FEBS Lett. **508**: 272-276, 2001.

Tamura, K., Dudley, J., Nei, M., Kumar, S.: *MEGA4*: molecular evolutionary genetics analysis (MEGA) software version 4.0. - Mol. Biol. Evol. **24**: 1596-1599, 2007.