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PHYLOGENETIC ANALYSIS OF SOME *Citrus* L. (RUTACEAE) TAXA IN TURKEY BASED ON CHLOROPLAST (cpDNA) *trn*L INTRON AND *trn*L-F DNA SEQUENCES

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In this study, phylogenetic analysis of some Turkish *Citrus* species was conducted based on chloroplast DNA (*trn*L intron and *trn*L-F) sequences. *Citrus* taxa were plotted on a phylogenetic tree where *Zanthoxylum ailanthoides* was used as the outgroup.The sequences for *trn*L intron and *trn*L-F regions of the outgroup were retrieved from NCBI GenBank. All plant samples were collected from different locations during the inflorescence and vegetation periods, and brought to the laboratory. Genomic DNA was isolated from healthy green leaves using DNAeasy Plant Mini Kit. *trn*L intron region was amplified using universal primers *trn*c and *trn*d, while for the *trn*L-F region *trn*e and *trn*f were used. Later obtained DNA sequences were edited using BioEdit 7.0.4.1 and FinchTV. Sequencing data were aligned via ClustalW program and analyzed using MEGA 6.0 software. Maximum Likelihood and bootstrap trees were constructed in order to identify the relationships among *Citrus* taxa. The *trn*L intron sequences ranged from 554 to 581 nucleotides. The average nucleotide composition of *trn*L intron was 26.3% T, 16.8% C, 37.7% A and 19.2% G. The divergence values varied from 0.000 to 0.057. The *trn*L-F sequences ranged from 392 to 399 nucleotides. Average nucleotide composition was 34.3% T, 21.2% C, 27.4% A and 17.1% G, while the divergence values of *trn*L-F sequences varied from 0.000 to 0.003. As a result, trees constructed based on *trn*L intron region have systematically been found to show more reliable and compatible results than those formed based on the *trn*L-F region.

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INTRODUCTION

Citrus is a genus of family Rutaceae that comprises some 158 genera and 1900 species. It is mainly tropical to semi tropical in origin, and is assumed to have originated from the region within Northeast India, South China, Indonesia and Peninsular Malaysia (WALI *et al.*, 2013). It includes some of the major fruit crops of the world, such as the citrons, lemons, limes, mandarins, sour oranges, sweet oranges, pummelos, grapefruits, kumquats, etc. (KUMAR *et al.*, 2013). *Citrus* fruits and side products have high economical and medical values due to their multisided uses like food industry, cosmetics and folk medicine (SILALAHI, 2002; SAIDANI *et al.*, 2004). Various studies on *Citrus* shells have revealed the presence of significant components that can be used for the pharmacological or pharmaceutical purposes. A number of components with activities such as antioxidant, antimicrobial, anti-inflammatory and antiproliferative activities have been obtained from different shells (SAWALHA *et al.*, 2009; VELAZQUEZ *et al.*, 2013).

Molecular marker technology, which has been rapidly developing in the last 20 years, has introduced a number of new approaches to the genetic characterization of varieties, in chromosome mapping, in the characterization of gene sources (NAVAL *et al.*, 2010; PINAR *et al.*, 2017). Chloroplast markers are widely used in phylogenetic evaluations. The low evolutionary rate of chloroplast DNA is the biggest drawback for the investigation of intraspecific relationships among sample sets. On the other hand, noncoding DNA sequences of the chloroplast genome evolve rapidly, and present a valuable source for phylogenetic studies (TÜRKTAŞ *et al.*, 2012). Chloroplast genome non coding sequences including *trn*L (UAA) intron and the intergenic spacer *trn*L (UAA) 3' exon- *trn*F (GAA) have phylogenetic capacity to reveal phylogenetic relationships of intra-species to inter-family level (XU and BAN, 2004; LIU *et al.*, 2006; TSAI *et al.*, 2006; KOCAK *et al.*, 2018). In this study, sequence analysis of some *Citrus* L. taxa using *trn*L intron and *trn*L-F (chloroplast DNA) sequences was performed to elucidate phylogenetic relationships among the investigated taxa. *Zanthoxylum ailanthoides* was used as out-group.

MATERIALS AND METHODS

Plant Specimens and Genomic DNA Isolation

Citrus taxa used in the study were collected from certain regions in Aegean region (Aydın, Muğla, İzmir-Bergama, Manisa-Salihli) between April and August, 2018. Total genomic DNA was extracted using DNAeasy Plant Kit (GeneMark). DNA samples were stored at -20°C.

PCR Amplifications and Sequencing

We amplified double-stranded DNA of the complete *trn*L intron and *trn*L-F regions from each genomic DNA. Amplification of the whole *trn*L intron region was performed with primers trnc and *trn*d (TABERLET et al., 1991) (Table 1). The amplification process was performed in 25 µL PCR reaction. Each PCR reaction contained 1 µL *trn*c and 1µL for *trn*d primers (5 µM each), 5 µL Master mix, 2.0 µL total genomic DNA (10-50 ng), and 16 µL ddH_2O. PCR amplifications of trnL-F cpDNA were performed using the primers designed by TABERLET et al. 1991 (Table 1). The amplification process was performed in 25 µL PCR reaction. Each PCR reaction contained 2.0 µL of total genomic DNA (10-50 ng), 1.0 µL for

*trn*e primer, 1.0 µL for *trn*f primer, 5.0 µL of master mix and 16.0 µL ddH_2O. Table 2 shows the trnL intron and *trn*L-F PCR cycles with their respective conditions. Gel electrophoresis with 0.8% agarose gel run in 10X TBE (Tris-Borate EDTA) buffer was used to size fractionate amplicons. Subsequently, gels were stained with ethidium bromide and visualized on a UV trans-illuminator. The *trnc*/*trn*d and *trn*e/*trn*f primer pairs were used both for amplification and for sequencing which were conducted at Triogen Inc. (İstanbul, Turkey) using an ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, CA, USA) with a BigDye cycle sequencing kit (Applied Biosystems). For each sample, forward and reverse sequencing reactions were performed and the sequences were checked via GenBank (NCBI) through BLASTn search. Later obtained DNA sequences were edited both manually and by using BioEdit 7.0.4.1 (HALL, 1999) and FinchTV programs.

Table 1. trnL intron and trnL-F primers used in this study and their designers

Primer name	5' to 3' Primer sequence	Based on (the source publication)
Forward *trn*c (F)	CGA AAT CGGTAG ACG CTA CG	Taberlet et al., 1991
Reverse *trn*d (R)	GGGGATAGAGGGACTTGAAC	Taberlet et al., 1991
Forward *trn*Le	GGTTCAAGTCCCTCTATCCC	Taberlet et al., 1991
Reverse *trn*Ff	ATTTGAACTGGTGACACGAG	Taberlet et al., 1991

Table 2. Cycles and conditions of trnL intron and trnL-F PCR reactions

Pre-heating	94 °C	5 min	1 cycle
1. step	94°C	30 s	
2. step	50°C	30 s	
3. step	72°C	90 s	35 cycles
4. step	72°C	8 min	1 cycle
5. step	4°C	20 min	

Alignment and Phylogenetic Analysis

*trn*L intron and *trn*L-F sequences were aligned using ClustalW alignment software (THOMPSON et al., 1994). Ends of the alignment were trimmed to make all the sequences equal length which was 598 nucleotide positions in the final dataset for *trn*L intron region, and 399 for *trn*L-F. The phylogenetic tree generated based on Maximum Likelihood method was constructed using MEGA 6.0 software (TAMURA et al., 2013). The phylogenetic tree was

evaluated with bootstrap test based on 1000 replicates (FELSENSTEIN, 1985). Sequences of *Zanthoxylum ailanthoides* taxa used as outgroup in the tree were retrieved from NCBI (For *trn*L intron; MG975313.1, For *trn*L-F Gen Bank: HM851511.1).

RESULTS AND DISCUSSION

In this study, *trn*L intron sequences obtained ranged from 554 to 581 nucleotides among 12 specimens (only Turkish *Citrus* species). The highest number of nucleotides for the *trn*L intron sequence (581 bases) was observed in *Citrus reticulata* (Bergama population) while the lowest number of nucleotides for the *trn*L intron sequence (554 bases) was observed in *Citrus sinensis* (Muğla population). Average nucleotide composition of *trn*L intron was 26.3% T, 16.8% C, 37.7% A and 19.2% G. The maximum GC content (37.6%) and the lowest AT content (62.5%) were observed in *Citrus sinensis* (Muğla population) while the lowest GC content (35.5%) and the highest AT content (64.6%) were recorded in *Citrus reticulata* (Bergama population) (Table 3). The total length of the aligned *trn*L intron sequence matrix was 598 nucleotides. Genetic distance method based on *trn*L intron set was performed with MEGA 6.0 software. The divergence values differed from 0.000 to 0.057 (Table 4). For cpDNA *trn*L-F sequences, the average base length ranged from 392 to 399 nucleotides among 12 specimens (Except for outgroup *Zanthoxylum ailanthoides*).

Table 3. Length, A+T content and G+C content of trnL intron sequences of Citrus taxa

Taxa	*trn*L intron (bp)	A (%) content	T (%) content	G (%) content	C (%) content	A+T (%) content	G+C (%) content
C. paradisi (Aydın)	570,0	37,5	26,5	19,3	16,7	64	36
C. limon (Aydın)	575,0	37,9	26,4	19,0	16,7	64,3	35,7
C. reticulata (Aydın)	580,0	38,3	26,2	19,3	16,2	64,5	35,5
C. sinensis (Aydın)	575,0	37,7	26,4	19,1	16,7	64,1	35,8
C. aurantium (Aydın)	576,0	38,4	26,0	19,3	16,3	64,4	35,6
C. reticulata (Bergama)	581,0	38,6	26,0	19,1	16,4	64,6	35,5
C. sinensis (Bergama)	570,0	37,5	26,5	19,3	16,7	64	36
C. paradisi (İzmir)	577,0	37,8	26,3	19,1	16,8	64.1	35,9
C. limon (Muğla)	573,0	37,0	26,5	19,4	17,1	63,5	36,5
C. sinensis(Muğla)	554,0	36,3	26,2	19,0	18,6	62,5	37,6
C. sinensis (Salihli)	578,0	37,4	26,6	19,0	17,0	64	36
C. aurantium (Muğla)	566,0	37,6	26,3	19,3	16,8	63,9	36,1
Average	572,9	37,7	26,3	19,2	16,8	64	36

The highest number of nucleotides for the *trn*L-F sequences (399 bases) was observed in *Citrus sinensis* (Bergama population) while the lowest number of nucleotides for the *trn*L-F sequences (392 bases) was observed in *Citrus aurantium* (Muğla population). Average nucleotide composition of *trn*L-F sequences was 34.3% T, 21.2% C, 27.4% A and 17.1% G. The highest AT content (62.00%) and the lowest GC content (38.10%) was observed in *Citrus reticulata* (Bergama population) while the lowest AT content (61.60%) and

the highest GC content (38.4%) were recorded in *C. paradisi* (Aydın), *C. limon* (Aydın), *C. sinensis* (Aydın), *C. sinensis* (Bergama), *C. paradisi* (İzmir), *C. limon* (Muğla), *C. sinensis* (Muğla) and *C. sinensis* (Salihli) (Table 5). The total length of the aligned *trn*L-F sequence matrix was 399 nucleotides. Genetic distance method based on *trn*L-F set was performed with MEGA 6.0 software. The divergence values varied from 0.000 to 0.003 (Table 6).

Table 4. Pairwise distances among some Citrus taxa based on trnL intron sequences obtained using MEGA 6.0 software distance matrix

Taxa	1	2	3	4	5	6	7	8	9	10	11	12
C. paradisi (Aydın)												
C. limon (Aydın)	0,000											
C. reticulata (Aydın)	0,004	0,004										
C. sinensis (Aydın)	0,000	0,000	0,004									
C. aurantium (Aydın)	0,004	0,004	0,000	0,004								
C. reticulata (Bergama)	0,004	0,004	0,000	0,004	0,000							
C. sinensis (Bergama)	0,000	0,000	0,004	0,000	0,004	0,004						
C. paradisi (İzmir)	0,017	0,017	0,021	0,017	0,021	0,021	0,017					
C. limon (Muğla)	0,009	0,009	0,013	0,009	0,013	0,013	0,009	0,015				
C. sinensis (Muğla)	0,053	0,053	0,057	0,053	0,057	0,057	0,053	0,055	0,043			
C. sinensis (Salihli)	0,013	0,013	0,017	0,013	0,017	0,017	0,013	0,019	0,008	0,047		
C. aurantium (Muğla)	0,000	0,000	0,004	0,000	0,004	0,004	0,000	0,017	0,009	0,053	0,013	
Z.ailanthoides	0,057	0,057	0,061	0,057	0,061	0,061	0,057	0,075	0,067	0,111	0,071	0,057

Table 5. Length, A+T content and G+C content of cpDNA trnL-F sequences of Citrus taxa

Taxa	*trn*L-F(bp)	A (%) content	T (%) content	G (%) content	C (%) content	A+T (%) content	G+C(%) content
C. paradisi (Aydın)	396,0	27,5	34,1	17,2	21,2	61,6	38,4
C. limon (Aydın)	396,0	27,5	34,1	17,2	21,2	61,6	38,4
C. reticulata (Aydın)	395,0	27,3	34,4	17,0	21,3	61,7	38,3
C. sinensis (Aydın)	396,0	27,5	34,1	17,2	21,2	61,6	38,4
C. aurantium (Aydın)	396,0	27,5	34,3	16,9	21,2	61,8	38,1
C. reticulata (Bergama)	394,0	27,2	34,8	16,8	21,3	62	38,1
C. sinensis (Bergama)	399,0	27,6	34,1	17,3	21,1	61,7	38,4
C. paradisi (İzmir)	396,0	27,3	34,3	17,2	21,2	61,6	38,4
C. limon (Muğla)	396,0	27,3	34,3	17,2	21,2	61,6	38,4
C. sinensis (Muğla)	393,0	27,2	34,4	17,3	21,1	61,6	38,4
C. aurantium (Muğla)	392,0	27,3	34,4	16,8	21,4	61,7	38,2
C. sinensis (Salihli)	396,0	27,3	34,3	17,2	21,2	61,6	38,4
Avg.	395,4	27,4	34,3	17,1	21,2	61,7	38,3

Table 6. Pairwise distances among some Citrus taxa based on cpDNA trnL-F sequences obtained using MEGA 6.0 software distance matrix

Taxa	1	2	3	4	5	6	7	8	9	10	11	12
C. paradisi (Aydın)												
C. limon (Aydın)	0,000											
C. reticulata (Aydın)	0,003	0,003										
C. sinensis (Aydın)	0,000	0,000	0,003									
C. aurantium (Aydın)	0,003	0,003	0,000	0,003								
C. reticulata (Bergama)	0,003	0,003	0,000	0,003	0,000							
C. sinensis (Bergama)	0,000	0,000	0,003	0,000	0,003	0,003						
C. paradisi (İzmir)	0,000	0,000	0,003	0,000	0,003	0,003	0,000					
C. limon (Muğla)	0,000	0,000	0,003	0,000	0,003	0,003	0,000	0,000				
C. sinensis (Muğla)	0,000	0,000	0,003	0,000	0,003	0,003	0,000	0,000	0,000			
C. aurantium (Muğla)	0,000	0,000	0,003	0,000	0,003	0,003	0,000	0,000	0,000	0,000		
C. sinensis (Salihli)	0,000	0,000	0,003	0,000	0,003	0,003	0,000	0,000	0,000	0,000	0,000	
Z.ailanthoides	0,097	0,097	0,100	0,097	0,100	0,100	0,097	0,097	0,097	0,097	0,097	0,097

By assigning a taxonomic rank to a group of organisms, taxonomists aim to provide information about the nature of the phylogenetic relationships between such groups. Unfortunately, however, the criteria used to assign taxonomic rank are sometimes unclear because they are not always explicitly described. Usually, authors justify their conclusions about the taxonomic status of the groups of organisms under study by referring to, for example, ecological factors, morphological characters or genetic ones (WHANG *et al.*, 2002). Maximum Likelihood (ML) tree was formed based on *trn*L intron sequences of certain *Citrus* species distributed in Turkey, and the sequences of outgroup were taken from NCBI (Gen Bank). The ML tree formed based on *trn*L intron sequences belonging to the samples taken in Turkey consists of two large clades. Clade 1 was supported with 74% of bootstrap value and divided into four subclades. Subclade A, consists of *Citrus aurantium* (Aydın population), *Citrus reticulata* (Bergama population), *Citrus reticulata* (Aydın population) and *Citrus aurantium* (Muğla population), Subclade B, consists of *Citrus paradise* (Aydın population) and *Citrus limon* (Aydın population), Subclade C, consists of only *Citrus sinensis* (Aydın population) and Subclade D, consists of only *Citrus sinensis* (Bergama population). Clade 2 is a monophyletic group consisting of *Citrus sinensis* (Salihli population), *Citrus sinensis* (Muğla population), *Citrus limon* (Muğla population) and *Citrus paradise* (İzmir population) species. This group has a bootstrap value of 97% (Fig. 1).

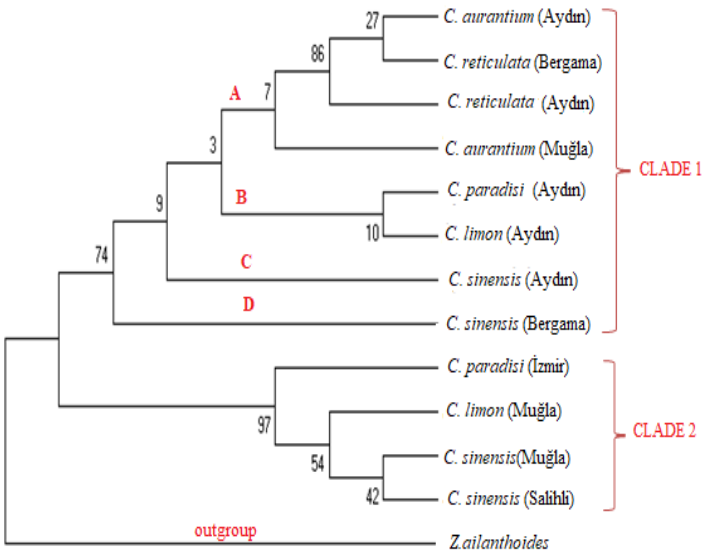

Fig. 1: Phylogenetic tree of *Citrus* ssp. *trn*L intron sequences constructed using Maximum likelihood method with MEGA 6.0

Maximum Likelihood tree was formed based on *trn*L-F sequences of certain *Citrus* species spread within Turkey and sequences of outgroup were taken from NCBI (GenBank). *Citrus* taxa consist of two clades in the ML tree formed based on *trn*L-F sequences of the specimens. Clade 1 is divided into four subclades. Subclade A consists of *Citrus paradise* (Aydın population), *Citrus limon* (Aydın population), *Citrus sinensis* (Aydın population) and *Citrus sinensis* (Bergama population) populations; Subclade B consists of *Citrus reticulata* (Aydın population), *Citrus reticulate* (Bergama population) and *Citrus aurantium* (Aydın population) with a bootstrap value of 64%; Subclade C consists of only *Citrus paradise* (İzmir population); and Subclade D consists of only *Citrus limon* (Muğla population). Clade 2 consists of *Citrus sinensis* (Muğla population), *Citrus aurantium* (Muğla population) and *Citrus sinensis* (Salihli popultion) (Fig. 2). However, phylogenetic tree formed based on *trn*L-F sequences was found to be different from the phylogenetic tree formed based on *trn*L intron sequences. *trn*L-F data were not resolved well, thus genetic relationship between the species could not be completely determined. Several studies were conducted in the past on the species of *Citrus* including RAPD and SCAR (COLETTA FILHO *et al.*, 1998; NICOLOSI *et al.*, 2000), ISSR (FANG and ROOSE, 1997), AFLP (PANG *et al.*, 2007), RFLP, *trn*D-*trn*T and *rbc*L-ORF 106 (JENA *et al.*, 2009). WALI *et al.* (2013) conducted the sequence analysis of the chloroplast *rps*14 gene on some species of *Citrus* and determined the phylogenetic relationship between the species. The genetic distance between the species were calculated to be between 0.000 and 0.404, and it was demonstrated that the *rps*14 gene didn't provide much phylogenetic information on the species of *Citrus.* SUN *et al.* (2015) performed the taxonomy and phylogenetic

analysis of the species of *Citrus* by using the core ITS regions. The study used 26 samples of 22 *Citrus* species. Although the ITS results didn't have any distinction in the tribe level, they provided the theoretical and experimental basis of the limitation of the species of *Citrus*. OUESLATI *et al*. (2016) determined the phylogenetic relations of 27 *Citrus* species growing in Tunisia by using the chloroplast *trn*L-F sequences. In the end of the study, they stated that the *trn*L-F sequence provided low genetic (nucleotide) diversity.

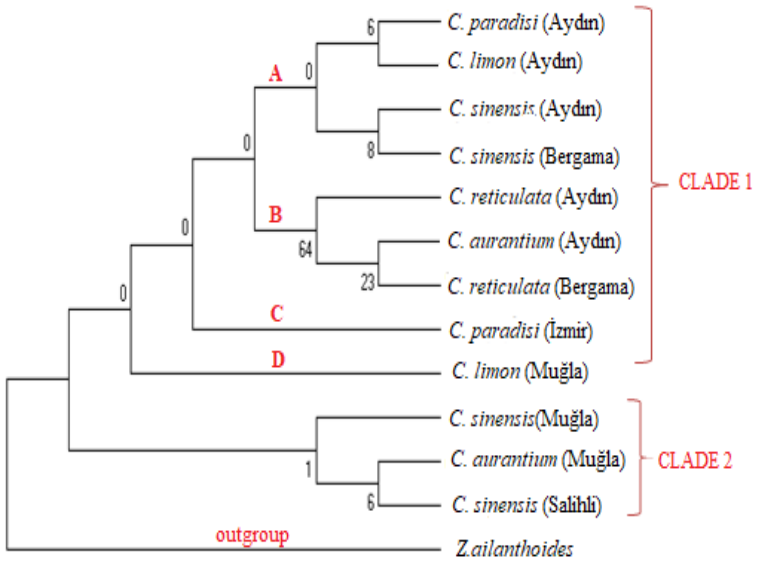

Fig. 2: Phylogenetic tree of *Citrus* ssp. *trn*L-F sequences constructed using Maximum likelihood method with MEGA 6.0

CONCLUSION

In this study, phylogenetic analysis of some Turkish *Citrus* L. taxa using *trn*L intron and *trn*L-F sequences was performed to elucidate phylogenetic relationships. Although there was a slight variation in the *trn*L intron sequences and a difference in the phylogenetic trees, there was no distinction in the phylogenetic trees obtained based on *trn*L-F sequences. In order to obtain more reliable phylogenetic results, different regions should be sequenced and different markers should be used. The data obtained by this study will shed light and be a resource for the future phylogenetic studies on both *Citrus* and different plant species.

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FILOGENETSKA ANALIZA NEKIH CITRUS L. (RUTACEAE) TAKSA U TURSKOJ NA OSNOVU INTRONA HLOROPLASTA (cpDNA) trnL-f I SEKVENCI DNK

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Izvod

U radu je rađena filogenetska analiza nekih *Citrus* vrsta u Turskoj, na osnovu sekvenci DNK hloroplasta (*trn*L intron and *trn*L-F). Urađeno je filogenetsko stablo *Citrus* taksona, gde je *Zanthoxylum ailanthoides* korišćen kao "*outgroup*". Sekvence za intron *trn*L i *trn*L-F regione preuzete su od NCBI GenBank. Svi biljni uzorci su sakupljeni sa različitih lokacija tokom vegetacionog perioda i cvetanja. Genomska DNK je izolovana iz zelenih listova upotrebom kita DNAeasy Plant Mini Kit. *trn*L region umnožen je korišćenjem univerzalnih prajmera *trn*c i *trn*d, dok su za *trn*L-F region korišćeni *trn*e i *trn*f. Sekvence su upoređivane korišćenjem programa ClustalW i analizirane pomoću softvera MEGA 6.0. *trn*L intron sekvence bile su od 554 do 581 nukleotida. Prosečna kompozicija nukleotida introna *trn*L bila je 26.3% T, 16.8% C, 37.7% A i 19.2% G. Divergentnost je varirala od 0.000 do 0.057. *trn*L-F sekvenca je bila u opsegu od 392-399 nukleotida, dok je prosečna nukleotidna kompozicija bila 34.3% T, 21.2% C, 27.4% A i 17.1% G, a nivo divergentnosti *trn*L-F sekvence bio je od 0.000 do 0.003. Kao rezultat, filogenetsko stablo konstruisano na osnovu regiona introna *trn*L, bilo je mnogo pouzdanije i sa kompatibilnijim rezultatima od stabla formiranog na osnovu regiona *trn*L-F.

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