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DNA Barcoding of *Liriomyza sylvatica* Sehgal 1971 (Diptera: Agromyzidae): a new record for Türkiye

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Abstract In this study, DNA barcoding of *Liriomyza sylvatica* Sehgal 1971 (Diptera: Agromyzidae) was collected from a forest area in Aydın, Türkiye between June and September 2023. Adults were obtained by culturing with galleried leaves in the laboratory. After the morphological diagnosis of *L. sylvatica*, HCO/LCO universal primers were used to reproduce the COI gene region of the samples, and 614 bp COI regions were obtained. Sequencing of the samples was performed at BM Labosis using ABI 3730XL DNA sequencer (Applied Biosystems). The primer pair used in PCR was also used in sequencing. As a result of the sequencing, files sequenced with both primers were compared with BioEdit software to create a single file. The obtained sequence was compared with the samples registered in the gene bank (NCBI, Nucleotide BLAST) to determine the species. The nucleotide distribution of the amplified COI region and Neighbour Joining (NJ) tree were calculated in the MEGA 11 package program. The control of whether the obtained sequence was pseudogene was also performed in the Gen Bank BlastX module. In our study, the insects obtained in Aydın/Türkiye were determined to *be L. sylvatica,* and Genbank access records were obtained. *L. sylvatica* is a new record for the Agromyzidae fauna of Türkiye.

Keywords COI, DNA barcoding, *Liriomyza sylvatica*, Türkiye

1. Introduction

The species of the Agromyzidae family are phytophagous and can cause damage to the plants the whole year. Their larvae can cause a loss of up to 98% of the leaf area by opening galleries between the two epidermises of the leaf and can also lead to decreases in the chlorophyll content of the plant [1]. Adults can cause also indirect harm through their egg-laying behavior and helping vectors by viruses [2]. There are approximately 2700 species of the Agromyzidae family in the world, belonging to 27 genera, whereas 776 species in Europe. Up to now, 208 species have been identified in Türkiye [3, 4, 5, 6]. The species identification of leafminer flies is made by using their external morphological features (color, hair distribution number, etc.) and aedeagus. The fact that they are very small in size and that the diagnostic features between species are very similar to each other, as well as the lack of sufficient experts in this field, necessitate turning to morphological techniques in diagnosis. The COI gene region can be used as an identification marker in studies conducted with insects as well as in most animal groups. It has many important advantages such as having extensive phylogenetic data and ease of operation when compared to other protein-coding genes [7]. For these reasons, this study aimed to perform molecular identification and determine phylogenetic distances of *Liriomyza sylvatica*, which is thought to be a new record for Türkiye, by gene barcoding.

2. Materials and Methods

Samples were collected by sweep netting in Aydın provinces, Türkiye (37°41′15′′N, 27°41′5′′E, 770m) between June and September 2023.

Laboratory studies

Species identifications were made according to Spencer [8]. and Sehgal [9]. Polymerase Chain Reaction (PCR) was used to amplify the COI gene region and this study was carried out with a PCR master mix and universal COI primer pair (LCO1490 and HCO2198). The PCR reaction was prepared in a total volume of 25 µL containing 2 µL template DNA and 12.5 µL PCR master mix. PCR master mix contained Tris-HCl pH 8.5, (NH4)2SO4, 4 mM MgCl2, 0.2% Tween 20, 0.4 mM each dNTP, Ampliqon Taq DNA polymerase, non-interacting red dye, and stabilizer. The PCR cycle was performed as indicated in Table 1.

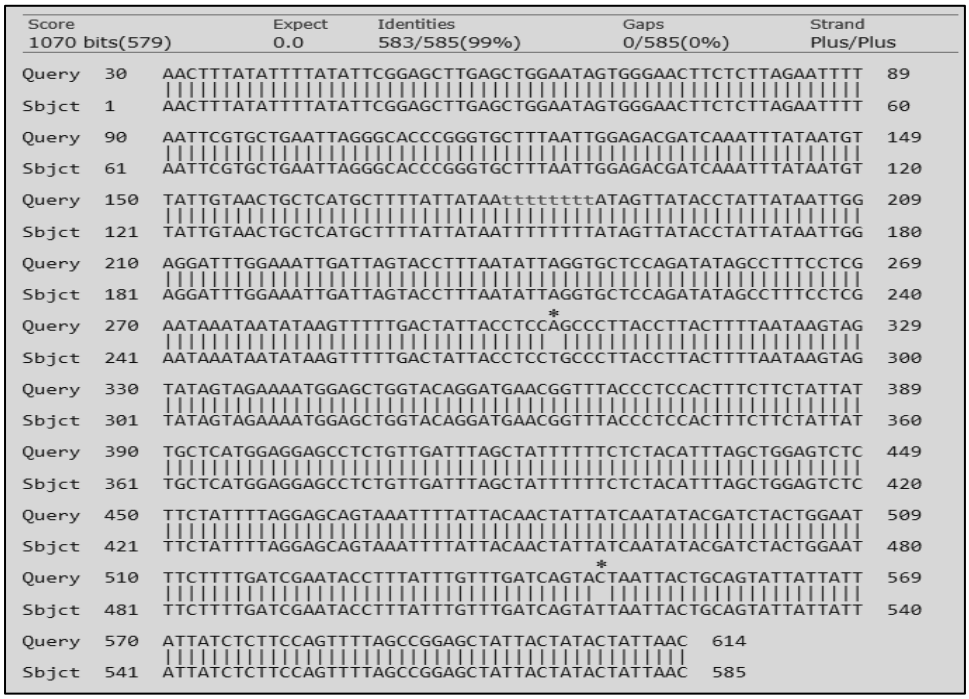
Table 1: COI gene region PCR conditions

Stages of PCR		**Temperature/Time**	**Cycle number**
Initial denaturation		95 °C / 5 min	1
Steps of cycles	Denaturation	95 °C / 30 sec	35
	Primer annealing	55 °C / 40 sec	35
	Extension	72 °C / 40 sec	35
Final extension		72 °C / 5 min	1

Sequencing of the samples was performed at BM Labosis using ABI 3730XL DNA sequencer (Applied Biosystems). The primer pair used in PCR was also used in sequencing. As a result of the sequencing, files sequenced with both primers were compared with BioEdit [10] software to create a single file. The obtained sequence was compared with the samples registered in the gene bank (NCBI, Nucleotide BLAST) to determine the species. The nucleotide distribution of the amplified COI region was calculated in the MEGA 11 [11]. package program. The control of whether the obtained sequence was pseudogene was also performed in the Gen Bank BlastX module. The obtained sequence was registered in Gen Bank and under the accession codes PQ182227. The partial COI sequence obtained from the study sample was aligned with the sequences of the related species *Liriomyza septentrionalis* (MG120229, MF636182), *L. phryne* (OK065481), *L. sylvatica* (KM854006, MF636607), *L. brassicae* (OR038460), *L. bryoniae* (OP161993), *L. trifolii* (OR038586), *L. sativae* (OR038567) and *L. fricki* (MF635341) in the Bioedit program to create a Neighbour Joining (NJ) [12] tree in the MEGA 11 package program [11] based on Kimura 2- parameter distances, with 1000 replicates of bootstrapping [13]. *Phytomyza gymnostoma* (MN943089) was used as an outgroup to root the tree.

3. Results & Discussion

The sequences read with both primers were aligned by eye in the Bioedit program.



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Score                Expect     Identities          Gaps          Strand
1070 bits(579)       0.0        583/585(99%)        0/585(0%)     Plus/Plus

Query  30   AACTTTATATTTTATATTCGGAGCTTGAGCTGGAATAGTGGGAACTTCTCTTAGAATTTT  89
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    AACTTTATATTTTATATTCGGAGCTTGAGCTGGAATAGTGGGAACTTCTCTTAGAATTTT  60

Query  90   AATTCGTGCTGAATTAGGGCACCCGGGTGCTTTAATTGGAGACGATCAAATTTATAATGT  149
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AATTCGTGCTGAATTAGGGCACCCGGGTGCTTTAATTGGAGACGATCAAATTTATAATGT  120

Query  150  TATTGTAACTGCTCATGCTTTTATTATAAttttttttATAGTTATACCTATTATAATTGG  209
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TATTGTAACTGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCTATTATAATTGG  180

Query  210  AGGATTTGGAAATTGATTAGTACCTTTAATATTAGGTGCTCCAGATATAGCCTTTCCTCG  269
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AGGATTTGGAAATTGATTAGTACCTTTAATATTAGGTGCTCCAGATATAGCCTTTCCTCG  240

Query  270  AATAAATAATATAAGTTTTTGACTATTACCTCCAGCCCTTACCTTACTTTTAATAAGTAG  329
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  241  AATAAATAATATAAGTTTTTGACTATTACCTCCTGCCCTTACCTTACTTTTAATAAGTAG  300

Query  330  TATAGTAGAAAATGGAGCTGGTACAGGATGAACGGTTTACCCTCCACTTTCTTCTATTAT  389
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TATAGTAGAAAATGGAGCTGGTACAGGATGAACGGTTTACCCTCCACTTTCTTCTATTAT  360

Query  390  TGCTCATGGAGGAGCCTCTGTTGATTTAGCTATTTTTTCTCTACATTTAGCTGGAGTCTC  449
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TGCTCATGGAGGAGCCTCTGTTGATTTAGCTATTTTTTCTCTACATTTAGCTGGAGTCTC  420

Query  450  TTCTATTTTAGGAGCAGTAAATTTTATTACAACTATTATCAATATACGATCTACTGGAAT  509
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  TTCTATTTTAGGAGCAGTAAATTTTATTACAACTATTATCAATATACGATCTACTGGAAT  480

Query  510  TTCTTTTGATCGAATACCTTTATTTGTTTGATCAGTACTAATTACTGCAGTATTATTATT  569
            |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  481  TTCTTTTGATCGAATACCTTTATTTGTTTGATCAGTATTAATTACTGCAGTATTATTATT  540

Query  570  ATTATCTCTTCCAGTTTTAGCCGGAGCTATTACTATACTATTAAC  614
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  ATTATCTCTTCCAGTTTTAGCCGGAGCTATTACTATACTATTAAC  585
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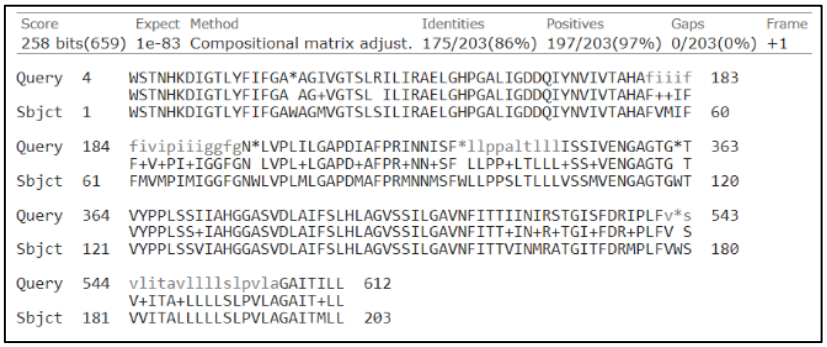
Figure 1: Similarity diagram with the sequence referenced as KM870016, which is one of the closest sequences to our sample sequence in the Genbank Nucleotide Blast model (symbol indicates dissimilar bases).*

The common sequence obtained is 614 base pairs long. In this sequence, the percentage equivalent of nucleotide frequencies was determined as T 40.1%, C 15.6%, A 28.2% and G 16.1%. The G+C ratio of the sequence is 31.76%. When compared with previously identified samples recorded in the BlastN module in the Genbank, the 6 *Liriomyza sylvatica* sequences (KM870016, KM865480, KM854006, MF636607, MF635288, and MF632746) with the highest similarity were at the top. The highest similarity was 99.66% with KM870016 and KM865480 sequences (Figure 1). Although the similarity with the MF632746 reference sequence was 99.80%, this sequence was not evaluated because it was short (510 bp) and one of the 2 base differences in our sample did not cover the query made with this sequence. As seen in the figure, there are 2 dissimilar bases (303: A>T and 547: C>T) between the sequence of our sample and the reference sequence, and there is no gap. Whether the sequence of our sample is a pseudogene was tested in the BlastX module of the Genbank.

BlastX comparison showed that the sequence we had was not a pseudogene, but a partial gene encoding a part of the cytochrome oxidase enzyme (Figures 2, 3).



Score	Expect	Method	Identities	Positives	Gaps	Frame
258 bits(659)	1e-83	Compositional matrix adjust.	175/203(86%)	197/203(97%)	0/203(0%)	+1

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Query  4    WSTNHKDIGTLYFIFGA*AGIVGTSLRILIRAELGHPGALIGDDQIYNVIVTAHAfiiif  183
            WSTNHKDIGTLYFIFGA AG+VGTSL ILIRAELGHPGALIGDDQIYNVIVTAHAF++IF
Sbjct  1    WSTNHKDIGTLYFIFGAWAGMVGTSLSILIRAELGHPGALIGDDQIYNVIVTAHAFVMIF  60

Query  184  fivipiiiggfgN*LVPLILGAPDIAFPRINNISF*llppaltlllISSIVENGAGTG*T  363
            F+V+PI+IGGFGN LVPL+LGAPD+AFPR+NN+SF LLPP+LTLLL+SS+VENGAGTG T
Sbjct  61   FMVMPIMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPSLTLLLVSSMVENGAGTGWT  120

Query  364  VYPPLSSIIAHGGASVDLAIFSLHLAGVSSILGAVNFITTIINIRSTGISFDRIPLFv*s  543
            VYPPLSS+IAHGGASVDLAIFSLHLAGVSSILGAVNFITT+IN+R+TGI+FDR+PLFV S
Sbjct  121  VYPPLSSVIAHGGASVDLAIFSLHLAGVSSILGAVNFITTVINMRATGITFDRMPLFVWS  180

Query  544  vlitavllllslpvlaGAITILL  612
            V+ITA+LLLLSLPVLAGAIT+LL
Sbjct  181  VVITALLLLLSLPVLAGAITMLL  203
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Figure 2: BlastX mapping diagram of our sample sequence.

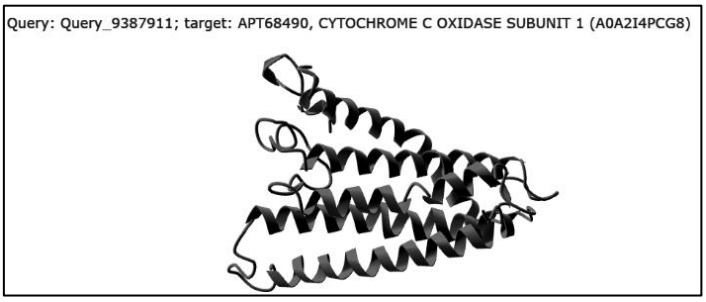

Figure 3: Partial cytochrome oxidase enzyme fragment encoded by the sequence.

In many previous studies on the COI gene region, the minimum distance between close species (sister group) within the genus in terms of the COI gene region was reported as greater than 2.5% [14, 15, 16, 17, 18, 19]. In this context, our sample was molecularly determined to be *Liriomyza sylvatica* in the light of the COI gene region. In our NJ tree, the genetic distance between the "*L. sylvatica*" pedigree and the "*L. septentrionalis*" and "*L. phyrne*" pedigrees in the sister group relationship is 9.6% and 7.9%. The genetic distance was 12.8% for "*L. trifoli*", 14.3% for "*L. brassicae*", 11.5% for "*L. sativa*" and 12.5% for "*L.fricki*".

Table 2: Estimates of Evolutionary Divergence over Sequence Pairs between Groups*

Species	[1]	[2]	[3]	[4]	[5]	[6]	[7]
***L. septentrionalis* [1]**							
***L. phryne* [2]**	0.091						
***L. sylvatica* [3]**	0.096	0.079					
***L. trifolii* [4]**	0.145	0.135	0.128				
***L. brassicae* [5]**	0.171	0.158	0.143	0.149			
***L. sativae* [6]**	0.138	0.124	0.115	0.070	0.135		
***L. fricki* [7]**	0.141	0.135	0.125	0.149	0.165	0.142	
***L. bryoniae* [8]**	0.148	0.151	0.128	0.126	0.146	0.120	0.135

*The number of base substitutions per site from averaging over all sequence pairs between groups is shown.

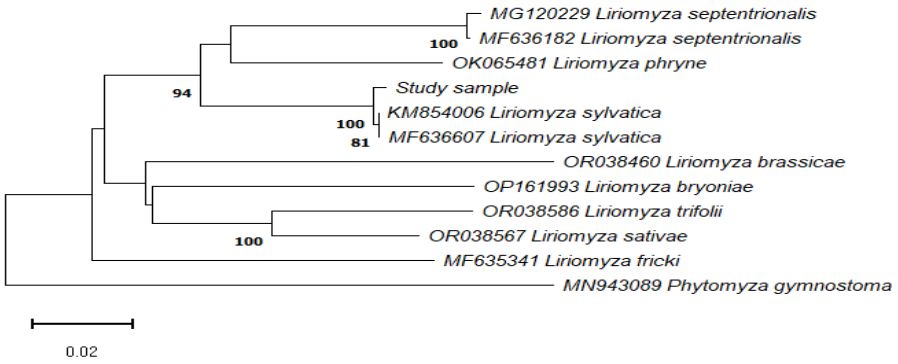

Figure 4: Phylogenetic analysis of samples

Studies on the use of COI gene region in the identification and phylogenetic analysis of species belonging to *Liriomyza* spp. have been increasing in recent years [20, 21]. This molecular method provides significant convenience, especially in the identification of species that are difficult to identify morphologically. With this study, *Liriomyza sylvatica* was recorded as a new record for Türkiye.

4. Conclusion

Due to reasons such as *Liriomyza* spp. being a small insect and the small number of experts working on this subject, DNA barcode studies can be used more effectively both for faster identification and to increase the reliability of the identification. In our study, the insects obtained in Aydın/Türkiye were determined to *be L. sylvatica*, and Genbank access records were obtained. *L. sylvatica* is a new record for the Agromyzidae fauna of Türkiye.

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