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MOLECULAR ANALYSIS OF WILDLY GROWN GENUS *VERONICA* SPECIES (PLANTAGINACEAE) IN IRAQ

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SUMMARY

Veronica is a prominent genus belonging to the family Plantaginaceae. Six species samples came from different mountainous areas of AL-Sulaimaniyah, Northern Iraq, comprising *V. anagalloides, V. anagallis-aquatica, V. beccabunga, V. minuta, Veronica persica,* and *V. polita*. DNA genomic manually extracted from plant leaves, and the specific PCR fragments partially covering the internal transcribed spacer-1, internal transcribed spacer-2, and 5.8S rRNA, and internal transcribed spacer-2 sequences were options for the latest study that proceeded with primers ITS5 (5' GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') to amplify the samples of six different *Veronica* species. The electrophoresis migration for PCR analysis showed that the length of the amplified segment was about 600 bp for all the studied samples. An assumption from the generated tree was that the detected nucleic acid variants showed a noticeable effect on changing the evolutionary positioning of the investigated samples compared with the wild-type sequences. All the investigated rRNA sequences deposited in the NCBI web server acquired unique accession numbers for the analyzed S1 to S3 sequences. The deposited sequences received the GenBank accession numbers, i.e., OP363796.1, OP363795.1, OP363794.1, OP363793.1, OP363792.1, and OP363791.1, representing the six investigated isolates of the *Veronica* species, respectively.

Keywords: *Veronica* species, Plantaginaceae, ITS primers, DNA sequences, genetic variations

Key findings: The variation of the rRNA sequences can also help in *Veronica* characterization due to its possible ability to adapt to variable genetic diversity. The sequencing reactions indicated the precise identity after performing NCBI BLASTn for these PCR amplicons. Concerning the investigated ribosomal amplicons, the NCBI BLASTn engine showed 99% to 100% sequence similarities between the sequenced samples and the intended reference target sequences.

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Interestingly, the alignment results of the ribosomal samples revealed the four nucleic acid variants represented by four nucleic acid substitutions in the analyzed S1 sample compared with the most similar referring reference nucleic acid sequences. These variations were g.93T>C, g.99A>T, g.108T>A, and g.114A>G compared with the referring sequences of *V. persica*.
he water deficit condition at the flowering stage caused a significant reduction in yield and its components in maize. The silicon treatment 6 mM L^{-1} concentration notably enhanced the grain and oil yields and carbohydrates.

INTRODUCTION

Veronica is the most famous genus of the flowering plant family Plantaginaceae, comprising 94 genera and 1,900 species (Albach *et al.,* 2004a, b). This family has a widespread distribution over most of the Northern Hemisphere and in many parts of the Southern Hemisphere and is ecologically highly diverse, with species growing in different habitats and elevations, from aquatic to even dry steppe from sea level to high alpine regions (Ellmouni *et al.,* 2018).

For over 150 times from their first collection made by Banks and Solander (1769), the common name of *Veronica* are speedwell, bird's eye, and gypsyweed; *Veronica* of Southern Hemisphere species is now the Hebe complex (Heads, 1992). Although *Hebe* naming beforehand was in 1789, it received little acceptance until Pennell (1921), Oliver (1925), Cockayne and Allan (1926), and Cockayne (1929). However, following the acceptance of *Hebe* by New Zealand authors, the placement of several *Veronica*-like species became problematic.

These species' morphological characters seem to place them close to Northern Hemisphere *Veronica*. However, their chromosome structure suggests a relationship with *Hebe*. Allan (1939) placed lesser weight on the cytological substantiation; however, Oliver (1944) erected a new rubric *Parahebe* and revitalized Hooker's *Pygmea* to accommodate some of these shops. Moreover, the affection for *Veronica*-like shops in Australia has also been unclear (Briggs and Ehrendorfer, 2006a, b).

The mentioned genus has many pharmacological benefits, with some *Veronica* species used in traditional medicines for the treatment of influenza, respiratory diseases, hemoptysis, laryngopharyngitis, cough, hernia, cancer, edema, and wound healing (Xue *et al.,* 2019). Therefore, various past studies refer to the genus that still receive scrutiny in modern biological and biochemical implications. Various molecular methods have proceeded recently to determine the genetic and phylogenetic relationships among and within species belonging to this genus, such as conventional (PCR) direct markers or PCR-dependent markers, viz., DNA sequencing for selected genes and DNA fragments (Albach *et al.,* 2009).

Three or four species of *Veronica* were specimens in phylogenetic analyses (Mu ¨ller and Albach, 2010). These studies also supported the close relationship among Asian countries like Chinese and Japanese species (Bentham, 1846; Rompp, 1928). The 19 species of *Veronica* are endemic, and most of them remained within China and adjacent regions belonging to just two subgenera; one is the subgenus, *Veronica,* with 25 to 30 species in alpine areas across Eurasia, a young group in the genus *Veronica* (Albach *et al.,* 2009).

One of the most famous sequences for phylogenetic inference at plant generic levels is the internal transcribed spacer (ITS) region of the 18S–5.8S–26S nuclear ribosomal cistron (Alvarez and Wendel, 2003).

The current study aimed at a molecular comparative review of the phenotypic patterns of *Veronica* species according to the hypothesis of evolution. It also sought to provide a first approximation of the evolutionary relationship among these species. In addition, the research hoped to generate accurate data about the genetic sequences of *Veronica* species wildly grown in Iraq.

MATERIALS AND METHODS

Genetic material and sampling

Veronica's six species collection of samples (*V. persica*, *V. anagallis-aquatica, V. beccabunga, V. polita, V. anagalloides*, and *V. minuta*) transpired during the period from March to May 2022 in different mountain areas of AL-Sulaimaniyah, Northern Iraq (between latitude 35° 62.38725' N and longitude 45°94.91482' E). These samples, collected from specific regions, are also suggestions in other studies (Al-Hadeethi *et al.,* 2019; Al-Hadeethi *et al.,* 2021; Al-Hadeethi *et al.,* 2022).

DNA extraction

Whole genomic DNA extraction from fresh plant leaves of the *Veronica* samples followed the manual extraction method. About 100 mg of leaves sustained the addition of liquid nitrogen, then ground with mortar and pestle. The transferred powder to a 15 mL tube received 1.5 mL of extraction buffer (100 mM Tris pH7.5, 1.5 M NaCl, 20 mM EDTA, 2% w/v CTAB, 2% w/v PVP-40, and 0.3% v/v mercaptoethanol) before incubation at 60 °C for one hour with shaking. Then, samples bore centrifugation for 5 min of 5000 g at 4 °C, with the supernatant transferred to a new tube. Isoamyl alcohol - chloroform (1:24 v/v) addition to samples incurred mixing for 5 min, then centrifuged for 10 min of 5000 g at 4 °C, and finally, transferring the supernatant to a new tube.

The addition of RNAse to a final concentration of 20 µg/mL continued to the samples' incubation at 37 °C for 15 min with shaking. Then, pour isoamyl alcohol - chloroform (1:24 v/v) into the samples received, mixing for 5 min, followed by a 10 min centrifuge of 5000 g at 4 °C. The supernatant, transferred to a new tube, acquired 1/2 volume of 5 M NaCl accompanied with gentle mixing. Adding three volumes of ice-cold 95% ethanol to the samples bore mixing for 2 min, followed by incubation at -20 °C for one hour. Then, samples' centrifugation ran for 10 min of 5000 g at 4 °C, with the supernatant discarded and samples washed with 3 mL of ice-cold 70% ethanol. Finally, the *Veronica* species samples underwent centrifugation for 10 min of 5000 g at 4 °C, discarding the ethanol by leaving tubes open to evaporate all ethanol. Then, DNA resuspension in 75 µl of 37 °C TE buffer occurred. The DNA quality and quantity measurement used a nanodrop spectrophotometer (more than 1.7) and then electrophoresed with agarose gel (Al-Anbari *et al.,* 2017; Dudai *et al.,* 2018).

PCR amplification

Specific PCR fragments partially covering the internal transcribed spacer-1, internal transcribed spacer-2, and 5.8S rRNA, and internal transcribed spacer-2 sequences were options for the presented study employed primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5' GGAAGTAAAAGTCGTAACAAGG-3') to amplify six *Veronica* species samples (assigned S1 to S6) taken from six variable isolates (Cheng *et al.,* 2016).

Thermal cycling conditions involved an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s, and a final extensión at 72 °C for 5 min. The PCR products incurred electrophoresis in 2% agarose gel in TBE buffer (Promega, USA). Running the gel at 120 V for one hour, it received staining with ethidium bromide, then photographed by a gel documentation system.

Sequencing methods

Nucleic acids sequencing

The amplified fragments' exposure to the Sanger dideoxy sequencing method (2000) revealed the patterns of their genetic polymorphism. Then, building two specific comprehensive trees helped identify the observed variants and their phylogenetic relationships. The resolved PCR amplicons sustained commercial sequencing from both termini (forward and reverse directions), following the instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). The obvious chromatographs obtained from ABI (Applied

Biosystems) sequence files received further analysis. Comparing the observed nucleic acid sequences of the local samples with the retrieved nucleic acid sequences helped identify the virtual positions and other details of the retrieved PCR fragments.

Interpretation of sequencing data

The sequencing results obtained from the PCR products of the targeted samples underwent editing, aligning, analyzing, and comparing with the respective sequences in the reference database using Bio Edit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). In each sequenced sample, the observed variations received a number in PCR amplicons and in their corresponding position within the referring genome. Each detected variant within the *Veronica* sequences' annotation used the Snap Gene Viewer ver. 4.0.4 (https://www.snapgene.com).

Deposition of sequences to GenBank

All the investigated and analyzed sequences proceeded to be submitted to the NCBI Bank portal following all the instructions described by the portal as detailed by the server (Benson *et al*., 2016). Each submitted sequence was in a nucleic acid sequence form in the NCBI to get unique GenBank accession numbers for the investigated sequences.

Comprehensive phylogenetic tree construction

In the presented study, the specific comprehensive tree construction applied the Neighbor-Joining protocol (Sarhan *et al.,* 2019). The observed variants' comparison with their neighbor homologous reference sequences used the NCBI-BLASTn server (Zhang *et al.,* 2000). Then, a full inclusive tree, including the observed variants, materialized by the neighbor-joining method and visualized as a circular cladogram using the iTOL suit (Letunic and Bork, 2019). Appropriate coloring in the comprehensive tree ensued to identify the sequences of each incorporated species.

RESULTS AND DISCUSSION

PCR analysis

In the PCR analysis, the results of electrophoresis migration showed that the length of the amplified segment was about 600 bp for the studied samples of the six *Veronica* species (Figure 1), which confirmed the conduct the subsequent tests.

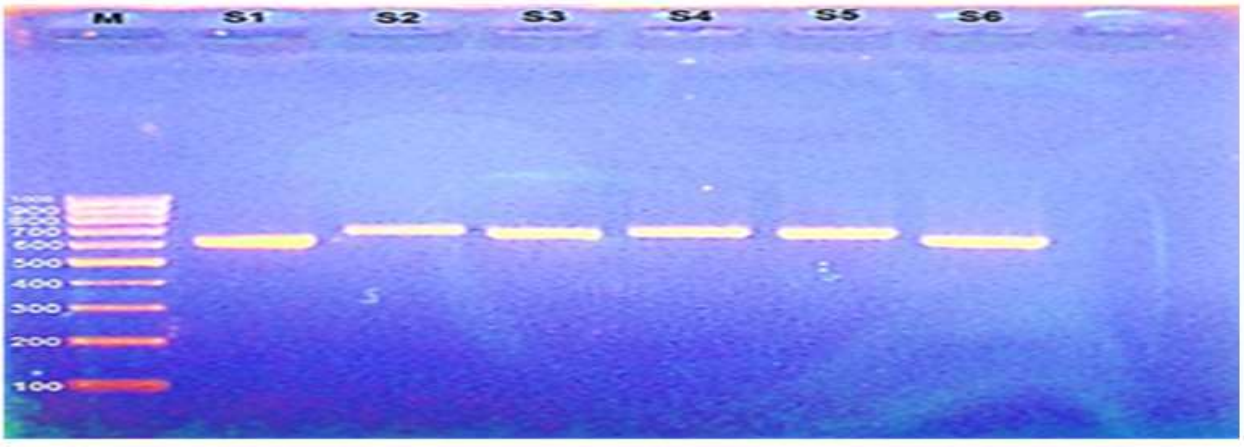

Figure 1. Agarose gel electrophoresis of PCR products of *Veronica* species. M = 100-bp ladder; S1 to S6 (600 bp) are the six *Veronica* species studied.

Sequencing results

Concerning the ribosomal amplicons, sequencing reaction results showed that the identified species of the studied S1 to S6 samples were *V. persica*, *V. anagallis-aquatica, V. beccabunga, V. polita, V. anagalloides*, and *V. minuta*, respectively. The results indicated the presence of four nucleic acid variants (g.93T>C, g.99A>T, g.108T>A, and g.114A>G) in the S1 sample, while the other five samples (S2 to S6) had alignment with their corresponding referring sequences with entire homology (GenBank Acc. No. KJ829444.1, KJ829470.1, MH711284.1, KJ829450.1, and ON202677.1, respectively). From the generated tree, an inference indicated that the identified nucleic acid variants showed a perceptible impact on changing the evolutionary placement of the investigated samples as compared with the wild-type sequences.

The altered S1 sample showed a positioning near several strains of *V. persica* isolated from the Asian and European variables. The neighbor phylogenetic distances in the tree further indicated a distinct biological diversity with the incorporated phylogenetic positioning of the S1 to S6 samples. The presented results were also analogous to past studies, which revealed that the recent phylogenetic analyses of the genus *Veronica* have also focused on the same species (Wagstaff and Garnock-Jones, 1998; Albach and Chase, 2001; Wagstaff *et al.,* 2002; Albach *et al.,* 2004a). These analyses have also shown the insufficiency of the last classifications to fix the problems in coherent evolutionary units; hence, the evolution of various species required reinterpretation (Albach *et al.,* 2004a, b, 2008).

Ribosomal nucleic acids sequencing

Within the studied locus, six samples comprised the presented study. Screening these samples succeeded in amplifying the rRNA sequences of the *Veronica* species specimens. Thus, the variation of the rRNA sequences can also serve for *Veronica* characterization due to its possible ability to adapt to variable genetic diversity. The sequencing reactions indicated the precise identity after performing NCBI BLASTn for these PCR amplicons. Concerning the investigated ribosomal amplicons, the NCBI BLASTn engine showed 99% to 100% sequence similarities between the sequenced samples and the intended reference target sequences. By comparing the observed nucleic acid sequences of the investigated *Veronica* species samples with the retrieved nucleic acid sequences (GenBank acc. AF509785.1, KJ829444.1, KJ829470.1, MH711284.1, KJ829450.1, and ON202677.1), the accurate positions and other details of the retrieved PCR fragments were distinct. The total length of the targeted locus was localized in the NCBI server, with the positions of the start and end of the targeted locus also authenticated within the most homologous target (Figure 2). After positioning the ribosomal amplicons' sequences within six species of *Veronica*, the conduct of genomic sequences followed. With their sequence details highlighted, the amplified amplicons' total length reached recognition.

Most past studies of DNA-based phylogenetic analyses exclusively depended on nuclear ribosomal ITS sequences, and Wagstaff and Garnock-Jones (1998) reported that four Australian species of *Veronica* showed a well-supported sister group relationship of the three out of four species with the New Zealand, with the *Veronica* (*Chionohebe*) *densifolia* also embedded among New Zealand species. Wagstaff *et al.* (2002) studied nine Australian species of *Veronica*, and seven of them again somewhat held up as a clade that was sister to the New Zealand Hebe. In contrast, analyzing three plastid rbcL sequences revealed the Australian species as a polyphyletic group.

Interestingly, the alignment results of the ribosomal samples revealed the four nucleic acid variants represented by four nucleic acid substitutions in the analyzed S1 sample compared with the most similar referring reference nucleic acid sequences. These variations were g.93T>C, g.99A>T, g.108T>A, and g.114A>G compared with the referring sequences of *V. persica* (GenBank Acc. No. AF509785.1). However, the other five isolates (*V. anagallis-aquatica, V. beccabunga,*

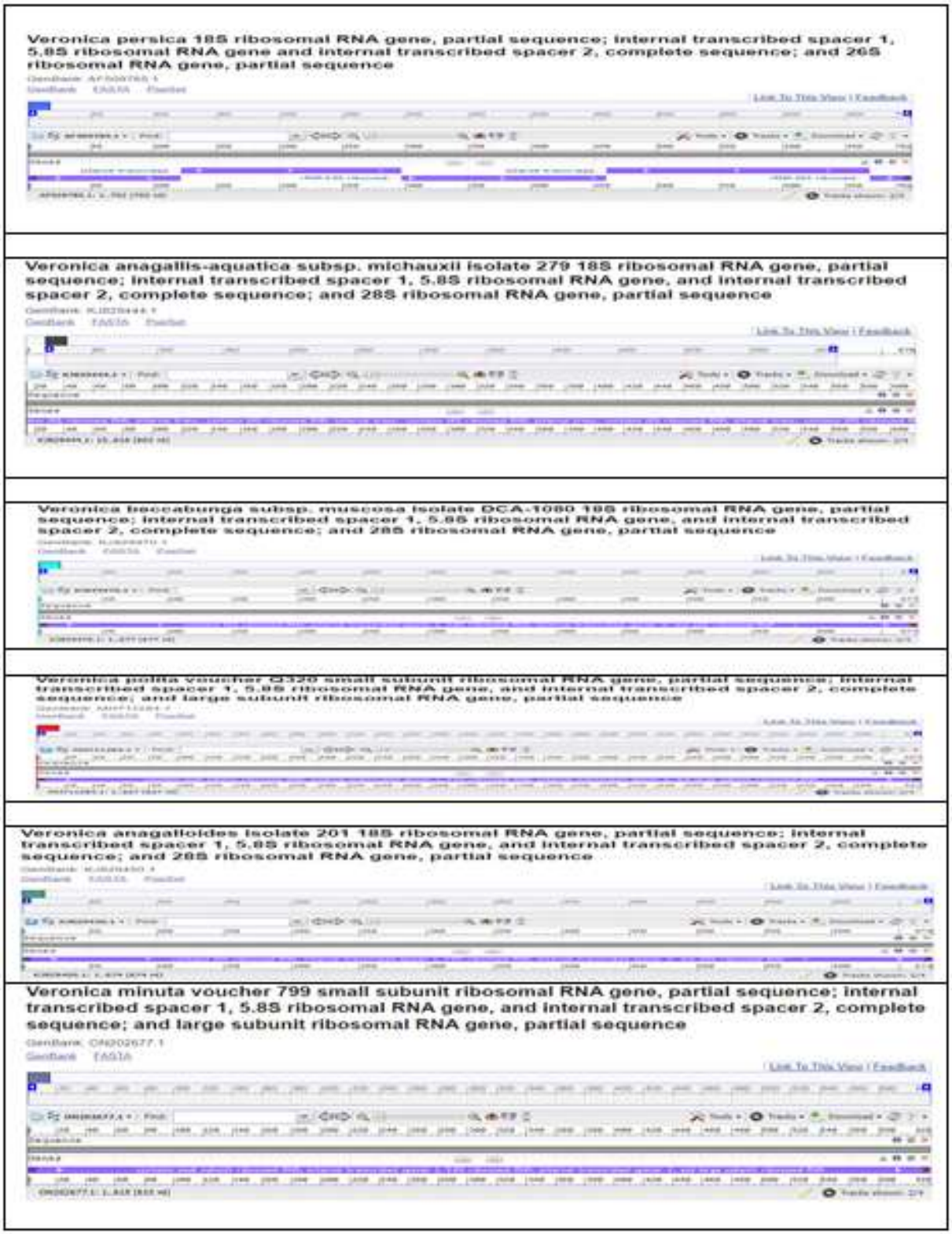

Figure 2. The positions of the retrieved ribosomal amplicons of six species belonging to the *Veronica* genus compared with their genomic sequences (GenBank acc. no. AF509785.1, KJ829444.1, KJ829470.1, MH711284.1, KJ829450.1, and ON202677.1).

V. polita, V. anagalloides, and *V. minuta*) did not reveal any variation concerning their corresponding DNA sequences (KJ829444.1, KJ829470.1, MH711284.1, KJ829450.1, and ON202677.1, respectively).

The pertinent study indicated the presence of four nucleic acid variants (g.93T>C, g.99A>T, g.108T>A, and g.114A>G) in the S1 sample, as also appearing in the DNA alignment chart. Confirming this kind of variation had the sequencing chromatograms of the investigated samples and their detailed annotations verified and documented, with the chromatograms of their sequences displayed according to their positions in the PCR amplicons sequencing results (Figure 3). All the investigated one rRNA sequences became submissions in the NCBI web server, obtaining unique accession numbers for the analyzed S1 to S3 sequences. The deposited sequences received the GenBank accession numbers, i.e., OP363796.1, OP363795.1, OP363794.1, OP363793.1, OP363792.1, and OP363791.1 representing the six investigated isolates.

The presented study indicated a complete phylogenetic tree generated according to nucleic acid variations observed in the amplified rRNA amplicons. All investigated samples were in alignment with other relative nucleic acid sequences. In this tree, the studied samples' incorporation alongside other relative sequences constituted six major clades of integrated sequences within the cladogram, namely, *V. persica, V. anagallis-aquatica, V. beccabunga, V. polita, V. anagalloides,* and *V. minuta*. The tree data indicated the presence of relatively high homology between three *Veronica* species (*V. persica, V. polita,* and *V. minuta*) incorporated clades within the same tree. Likewise, the two species (*V. anagallis-aquatica* and *V. beccabunga*) of other clades also emerged to have a high ratio of homology. However, the clade of *V. anagalloides* did not share close homology with any of the five incorporated clades with the phylogenetic tree of *Veronica*. The results further indicated the ability of rRNA sequence-based amplicons to distinguish the currently investigated *Veronica* sequences of the S1 to S6 samples without including any confusing interactions with other sequences of other species in the same genus. The total number of aligned nucleic acid sequences in this comprehensive phylogenetic tree was 36.

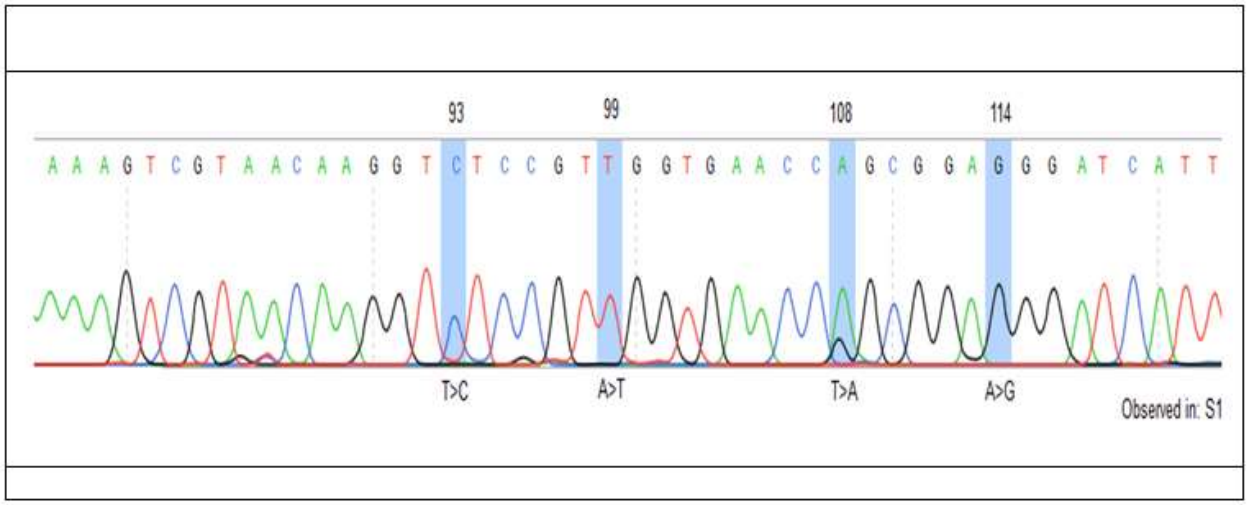

Figure 3. The chromatogram of the *Veronica persica* DNA sequences. The symbol ">" refers to the "substitution" variation detected.

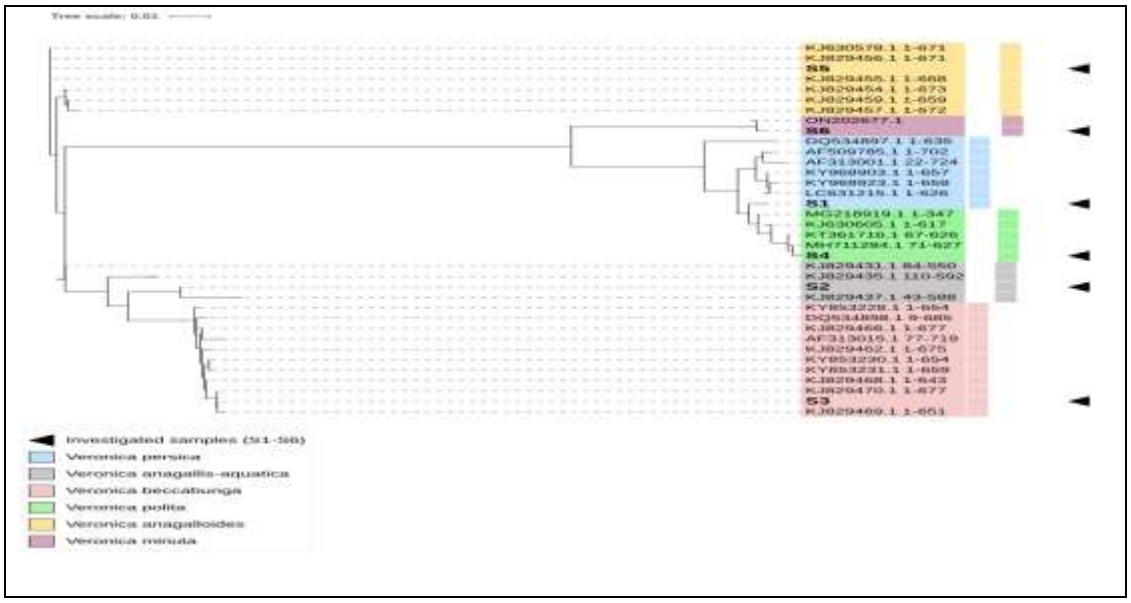

Figure 4A. A comprehensive rectangular cladogram phylogenetic tree of genetic variants of the rRNA sequences of six isolates of *Veronica*. The black-colored triangle refers to the analyzed variants. All the mentioned numbers referred to GenBank accession number of each referring species. The number "0.01" at the top portion of the tree refers to the degree of scale range among the comprehensive tree-categorized organisms. The letter "S#" refers to the code of the investigated sample.

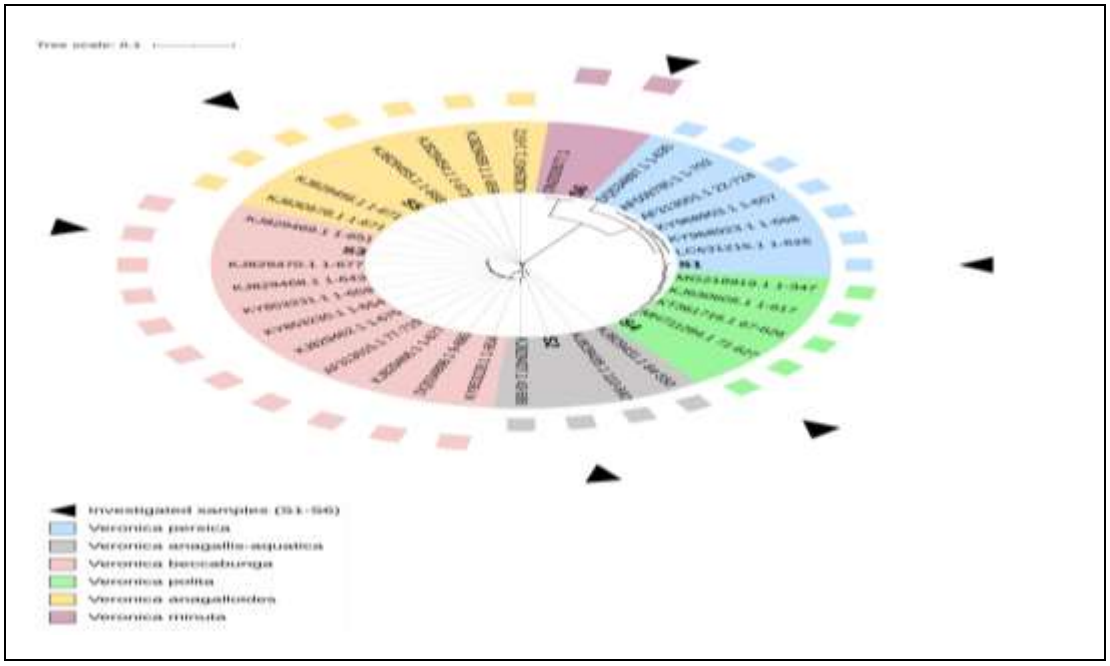

Figure 4B. A comprehensive circular cladogram phylogenetic tree of genetic variants of the rRNA sequences of six isolates of *Veronica*. The black-colored triangle refers to the analyzed variants. All the mentioned numbers referred to GenBank accession number of each referring species. The number "0.01" at the top portion of the tree refers to the degree of scale range among the comprehensive tree-categorized organisms. The letter "S#" refers to the code of the investigated sample.

As indicated above, the present studied *Veronica* samples incurred clustering into six phylogenetic clades of variable phylogenetic distances within six clades of *Veronica* sequences. The two types of cladograms generated explained three different representations of the incorporated sequences: a rectangular cladogram (Figure 4A) and a circular cladogram (Figure 4B). In both cases of the constructed cladograms, the studied samples' clustering into six main clades occurred within the *Veronica* sequences. In the assessed isolates, the most interesting fact signified the correlation of these with the ability of the utilized rRNA sequences-based amplicons to categorize the *Veronica* sequences into these observed phylogenetic distributions of *V. persica, V. anagallis-aquatica, V. beccabunga, V. polita, V. anagalloides,* and *V. minuta*.

Within the chief clade of *V. persica*, seven sequences of the same species reached integration. The S1 sample was suitable to various strains of the *V. persica* sequences that have been deposited from different origins, i.e., Japanese (GenBank Acc. No. LC631215.1), Chinese (GenBank Acc. No. KY968923.1 and KY968903.1), French (AF313001.1), and Austrian (GenBank Acc. No. AF509785.1). The presented sequences of the S1 sample exerted a remarkable tilt of phylogenetic distributions within this major clade. It might be due to the presence of four nucleic acid substitutions (g.93T>C, g.99A>T, g.108T>A, and g.114A>G) observed in this sample compared with the referring sequences of the species *V. persica*.

The S2 sample showed incorporation within the main clade of *V. anagallis-aquatica.* It was also close to one strain of the *V. anagallis-aquatica* sequences previously deposited from a Turkish origin (GenBank Acc. No. KJ829437.1). The S3 sample indicated inclusion within the prime clade of *V. beccabunga*, with suitability close to two strains of the *V. beccabunga* sequences with respective submissions from a Pakistani (GenBank Acc. No. KJ829469.1) and Kyrgyzstani (KJ829470.1) origins. The S4 sample's combination within the primary clade of *V. polita* revealed closeness to one strain of the *V. polita* sequences deposited earlier from a Chinese origin (GenBank Acc. No. MH711284.1).

The S5 sample became incorporated within the major clade of *V. anagalloides*, showing closeness to three strains of the *V. anagalloides* sequences earlier deposited from an Egyptian origin (GenBank Acc. No. KJ829455.1, KJ829456.1, and KJ630578.1). The S6 sample connected within the chief clade of *V. minuta.* Given the few deposited numbers of the ribosomal sequences of *V. minuta* in the NCBI database, the S6 sample emerged close to only one strain of the *V. minuta* sequences previously deposited from an Iranian origin (GenBank Acc. No. ON202677.1). However, no tilt was evident in the *Veronica* accession samples (S2–S6) from their corresponding phylogenetic positions due to the absence of nucleic acid variation compared with the referring sequences of these arrangements.

In detecting and discriminating such type of organism, interestingly, the utilized rRNA sequence-based amplicons provided the highest accuracy. The six major clades showed distinct positions without overlapping each other. These observations also indicated the ability of the utilized ribosomal sequences to differentiate the currently investigated flowering plants without any phylogenetic confusion. Furthermore, the tree also revealed that the *V. anagalloides* sequences have occupied ancestral positions, followed by *V. anagallis-aquatica* and *V. beccabunga,* respectively. The distinct evolutionary distances among the majority of the clades enunciated the highest resolution of the utilized PCR products of the rRNA sequences in detecting *Veronica* species.

The prevailing study findings also got support and validity from past findings by using plastid trnL-intron and trnL–trnF-spacer sequences, even though the resolution was low because of the low sequence variability (Albach *et al.*, 2005a). Moreover, the fused analysis of the ITS and trnL–F sequences by Albach *et al.* (2005b) also provided considerable support for the Monophyly of the Australian clade and even stronger support than in the analysis of ITS alone. Albach and Meudt (2010) also provided a similar pattern. Briggs and Ehrendorfer

Table 1. The pattern of the observed mutations in the amplified ribosomal sequences of *Veronica persica* in comparison with its corresponding sequences.

Sample	Native	Allele	Position	Variant type
S1	T	C	93	g.93T>C
S1	A	T	99	g.99A>T
S1	T	A	108	g.108T>A
S1	A	G	114	g.114A>G

(2006a, b) reported that the next branches in the ITS tree led to *V. notabilis,* and the Formosa clade, together with *V. lithophila* and *V. nivea*, the consistency of the Formosa clade was questionable; however, *V. formosa* and *V. nivea* also implied linkage with the plastid tree.

The latest results of the phylogenetic tree have also confirmed the sequencing reactions because they explained the actual neighbor-joining-based positioning in the observed nucleic acid variations. Noteworthily, the multiple European - Asian distribution of the significant investigated samples is undeniable. In turn, it gives further proof of the ability of the currently utilized ribosomal sequence-specific primers to describe the investigated *Veronica* sequences and their phylogenetic positions. Summarizing the obtained nucleic acid variations led to generating a variation table to describe all the nucleic acid variants observed and their positions in PCR fragments (Table 1).

CONCLUSIONS

The existing study explained the ability of the utilized rRNA sequences to detect the investigated *Veronica* species and discriminate them from each other. These ribosomal amplicons can efficiently help identify the biological diversity of a wider spectrum of *Veronica* DNA sequences. These promising amplicons can also serve to determine the further details within these identified *Veronica* sequences in various biological and geographical sources.

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