Identification and genetic characterization of a new totivirus from Bursera graveolens in western Ecuador

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Abstract

The complete genomic sequence of a previously uncharacterized virus provisionally named Bursera graveolens totivirus 1 (BgTV-1) was obtained from *Bursera graveolens* (Kunth) Triana & Planch., a tree known as “palo santo” in Ecuador. The BgTV-1 genome is monopartite double-stranded RNA (dsRNA) and 4,794 nucleotides (nt) long (GenBank accession number ON988291). Phylogenetic analysis of the capsid protein (CP) and RNA-dependent-RNA-polymerase (RdRp) placed BgTV-1 in a clade with other plant-associated totiviruses. Amino acid (aa) sequence comparisons of BgTV-1 putative proteins showed the highest identities to those of taro-associated totivirus L (QFS21890.1-QFS21891.1) and Panax notoginseng virus A (YP_009225664.1- YP_009225665.1) with 51.4% and 49.8%, for the CP, and 56.4% and 55.2% for the RdRp, respectively. BgTV-1 was not detected in any of the two endophytic fungi grown in BgTV-1-positive *B. graveolens* leaves, suggesting a plant totivirus. Based on distinct host and low aa identities between the CP of BgTV-1 and counterparts from closest relatives, the virus described in this study should be assigned as a new member of the genus *Totivirus.*

full text

Bursera graveolens (Kunth) Triana & Planch. (Common names: palo santo (“holy stick”), sassafras or incense tree) belongs to the Burseraceae family. It is one of the most common native trees in tropical dry forests of Ecuador, including the Galapagos Islands [1]. The distribution of *B. graveolens* covers from Mexico to Peru [2]. The wood, which has an intense and characteristic scent, is commonly dried and burned as mosquito repellent. In addition, *B. graveolens* essential oils are used to mitigate different types of pain, such as arthritis, atherosclerosis, and rheumatism [3]. Currently, there is no available information about diseases affecting *B. graveolens* trees including potential viral pathogens. Instead, most research focuses on the properties of its essential oils and their medicinal use [4,5,6].

The family *Totiviridae* contains viruses typically associated with latent infections of their fungal or protozoal hosts [7]. Over the last few years, however, several totivirus-like genomes have been found associated with different hosts such as plants, insects, arthropods, and fishes [8,9,10,11]. Currently, there are five genera within the family *Totiviridae*: *Giardiavirus*, *Leishmaniavirus*, *Totivirus*, *Trichomonasvirus,* and *Victorivirus.*

Members of the genus *Totivirus* have non-enveloped isometric virions about ~40 nm in diameter with genomes consisting of monopartite double-stranded RNA (dsRNA) of 4.5 - 7 kb with two open reading frames (ORFs). ORF 1 encodes a putative capsid protein (CP) and ORF 2 codes for the putative RNA-dependent RNA polymerase (RdRp). The RdRp is expressed as a CP-Pol fusion protein mediated by a −1 ribosomal frameshift (FS) which is operated by a heptameric slippery site before the stop codon of ORF 1 and an essential pseudoknot structure [7].

In this communication, we describe the genomic characterization and phylogenetic relatedness of a previously uncharacterized totivirus from *B. graveolens*.

In 2020, leaves from a *B. graveolens* tree located in Prosperina, a sector on the western side of Guayaquil, Ecuador (-2.151364, -79.953438) showing virus-like symptoms including chlorotic mottle and curling were collected for examination. These leaves were used for dsRNA extraction following the protocol described by Morris and Dodds [12]. The dsRNA was subjected to cDNA library preparation and sent for high-throughput sequencing (HTS). Sequencing was done on a NextSeq 500 Illumina platform as single 75 bp reads.

A total of 14,607,877 sequence reads were obtained through HTS. Sequence data was analyzed using Next Generation Sequencing Analysis tools available in Geneious Prime® 2022.0.1. Raw sequences were trimmed for adapter removal and quality using the BBDuk plugin and *de novo* assembled using SPAdes. A total of 2,418 contigs were assembled from the sequence set. BLASTx search identified a 4,771 nt long contig showing sequence homology to several members of the genus *Totivirus*. This putative totivirus contig was assembled from 92,171 reads. No other virus-like contigs were identified in this sample.

To confirm HTS sequencing results, primers were designed for re-sequencing (whole genome coverage) and viral detection. Total RNA extraction and reverse transcription (RT) were done as described by Halgren et al. [13]. Polymerase chain reaction (PCR) was performed using a 2x GoTaq Green Master Mix (Promega, USA) in a 10 µl mixture containing, 0.2 µl of each primer (40 µM), 2 µl cDNA template, and 2.6 µl of DEPC water. PCR parameters were as follows: 94 °C for 4 min, 40 cycles [94 °C for 40 s, 55 °C for 40 s, and 72 °C for 60 s], and a final extension step of 5 min at 72 °C. PCR products were cloned and sequenced as described above. All primers used in this study were designed using Geneious Prime® 2022.0.1 and can be found in Online Resource 1. All PCR reactions were done in a MiniAmp Plus Thermal Cycler (Applied Biosystems) and PCR amplicons were visualized on 2% agarose gels using a UV transilluminator, Gel Doc XR (BIO-RAD).

The 5´ and 3´ terminals of the viral genome were obtained using the 5´/3´ RACE Kit, 2nd Generation (Roche, Germany). RACE reactions were carried out using dsRNA as the initial template. A mixture containing the dsRNA and random primers was heated at 98°C x 10 min and immediately placed on ice. The denatured mixture was used to perform a RT reaction using a SuperScript III reverse transcriptase kit according to manufacturer instructions (ThermoScientific). Amplicons were cloned using a pGEM-T-Easy Vector System (Promega, USA) and sequenced by Sanger in both directions.

The complete genomic sequence consisted of 4,794 nt in length (NCBI GenBank ON988291) with a G + C content of 43.1%. The 5' - 3' UTRs are 13- (5’-GAAAGATATCAAG-3’) and 11- (5’-ACCCACAGATC-3’) nt long, respectively. The genome presents two ORFs: ORF 1 (frame 2, at nt positions: 69-2,128) codes for a putative CP of 686 amino acids (aa) (77.9-kDa), while ORF 2 (frame 1 at nt positions: 2,179-4,737) encodes a putative RdRp of 852 aa (98.2-kDa). A putative slippery site formed by the heptamer GGGUUUU was identified at nt positions 2,068-2,074 (Fig. 1). This RNA feature suggests the translation of a fusion CP-RdRp protein of approximately 170 kDa through a -1 FS mechanism, as previously reported for totiviruses [7].

Phylogenetic analyses were done using the amino acid sequences of the CP, RdRp or the putative CP-RdRp fusion protein of the tentatively new totivirus and representative homologues of the family *Totiviridae* available in the NCBI database. Multiple sequence alignments were done using the Multiple Sequence Comparison by Log- Expectation (MUSCLE) method [14]. A maximum-likelihood phylogenetic tree was constructed using MEGA X with the best-fitted protein models (WAG+G+F), (LG+G+I+F) and (LG+G+I) for the CP, CP-RdRp, and RdRp, respectively with 1,000 bootstrap replicates [15]. The tree topologies obtained by the three phylogenetic analyses were similar showing the tentatively new totivirus in a clade with taro-associated totivirus L, Pterostylis totivirus, Panax notoginseng virus, loquat-associated totivirus 1, and peach-associated virus 2 (Fig. 2). The phylogenetic tree using the CP showed poorly supported bootstrap values compared to the trees based on the CP-RdRp or RdRp, suggesting that the CP sequence is more diverse and may need additional homologues to confidently resolve the closest evolutionary relationships of the newly discovered Bursera graveolens totivirus.

According to the species demarcation criteria for totivirus, viruses with less than 50% aa sequence identity and found in distinct hosts should be assigned to different species [7]. When both putative proteins of BgTV-1 were analyzed, aa identities with its closest relatives were in the 37 - 51% range for the CP, and 55 - 58% range for the RdRp (Online Resource 2). Based on the fact that the here-described totivirus was found in a distinct unrelated plant host, we infer that it represents a new member of the *Totivirus* genus, with taro-associated totivirus L, Panax notoginseng virus B, and Pterostylis totivirus as closest relatives. The new virus was provisionally named Bursera graveolens totivirus 1 (BgTV-1).

Twenty-two samples of *B. graveolens* collected in Prosperina near the area where it was first identified were screened for BgTV-1. The leaf surface of collected samples was sterilized with 5% sodium hypochlorite and 70% ethyl alcohol, subjected to total RNA extraction, and virus tested by RT-PCR, as described above.

BgTV-1 was detected in 10 out of 22 *B. graveolens* trees tested in this study. Plants positive for the virus included both symptomless and symptomatic. Hence, it was not possible to ascribe the observed symptoms to BgTV-1 infection.

In recent years, there have been numerous reports about totivirus-like sequences identified in plants [8,16,17,18]. Nevertheless, the *Totivirus* is still considered a genus of fungus-infecting viruses by the ICTV [7]. To investigate the existence of an endophytic fungal host for BgTV-1, tissue from four BgTV-1-infected trees was collected, sanitized, placed in Potato Dextrose Agar (PDA) and kept under controlled conditions (28°C and 12-h light/dark cycle per 6 days) to obtain fungal cultures. For the identification of fungal endophytes, grown mycelia were subjected to DNA extraction by the modified CTAB method [19] and PCR as described above, using internal transcribed spacer (ITS) primers: ITS1 / ITS4 [20]. PCR was validated using DNA from *Trichoderma* spp. as a positive control. PCR products were cloned and sequenced, as described above. Two fungal endophytes were identified: *Nigrospora sphaerica* and *Bipolaris sorokiniana*, however, BgTV-1 was not detected in any of them.

A direct relation between plants and totiviruses has been demonstrated in recent years with the publication of new viral reports [8,16,17,18]. The genetic closeness of BgTV-1 and plant-associated totiviruses was demonstrated by the phylogenetic analysis and sequence comparisons presented in this study. This is the first report of a virus associated with *B. graveolens*. Further studies should be conducted to investigate the transmission, pathogenicity and host range for this new totivirus.

Declarations

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Ethics approval

This research does not contain any essays involving human participants or animals and was directed under Genetic Resource Access Permit # MAE–DNB–CM–2018–0098 granted by the Department of Biodiversity of the Ecuadorean Ministry of the Environment.

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Conflict of interest declaration

The authors declare have no conflict of interest.

Author contributions

Study conception and design: Juan F. Cornejo-Franco and Diego F. Quito-Avila. Material preparation, data collection and analysis were performed by Juan F. Cornejo-Franco, Robert A. Alvarez-Quinto and Dimitre Mollov. The first draft of the manuscript was written by Juan F. Cornejo-Franco; all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The complete genome sequence reported here can be encountered in the GenBank under accession number: ON988291

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Figures

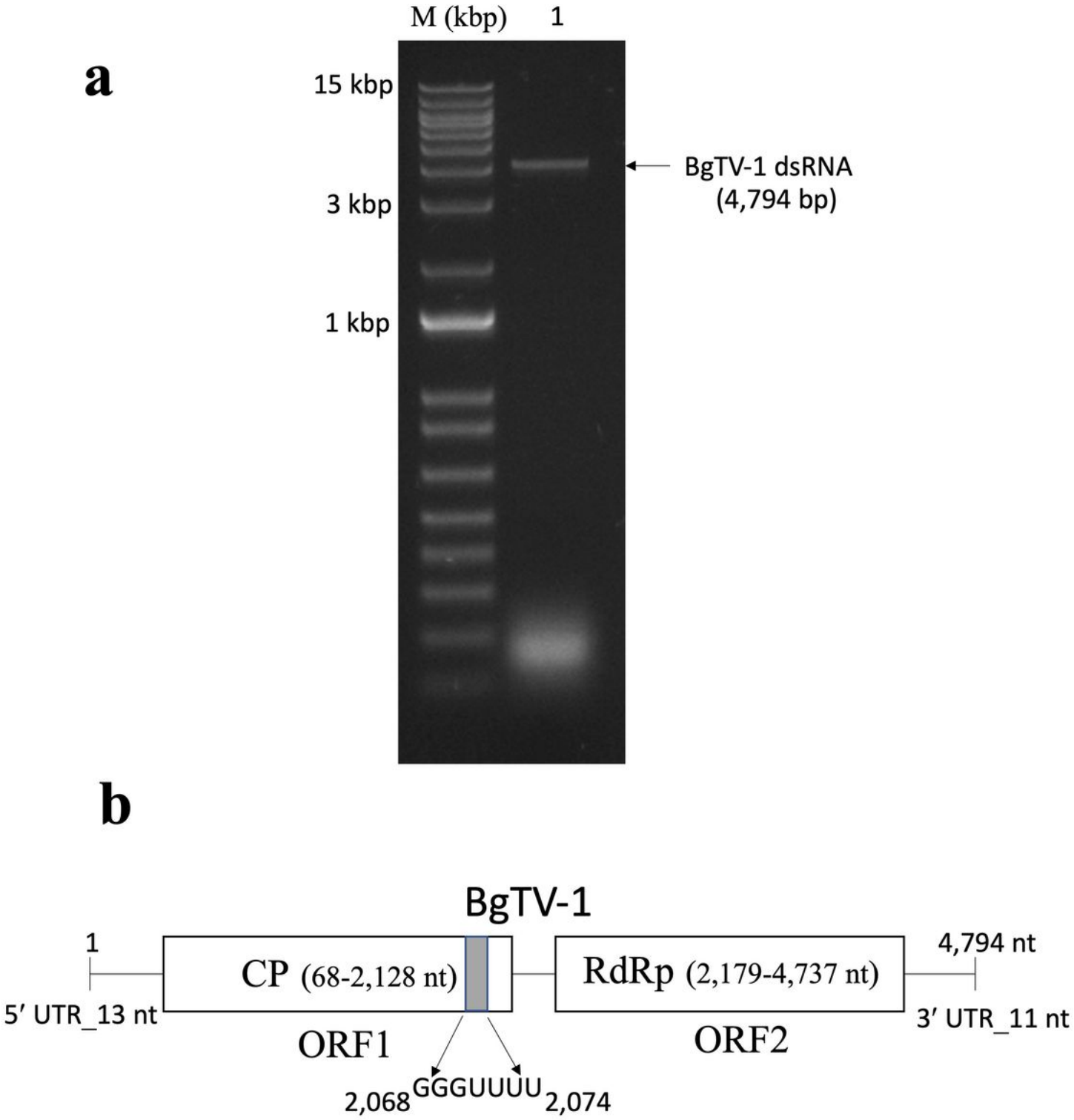

Figure 1

(A) Electrophoresis of double-stranded RNA (dsRNA) extracted from symptomatic leaves of *Bursera graveolens* in a 1% agarose gel. M, DNA marker (1 kb Promega); lane 1, dsRNA. (B) Schematic representation of the genomic organization of BgTV-1 with open reading frames (ORFs) indicated. CP: capsid protein, RdRp: RNA-dependent RNA polymerase, the 5'- and 3'- untranslated regions (UTR¢s), and

arrows indicate the putative slippery heptamer where the -1 frameshift is predicted to occur. The image is not drawn to scale.

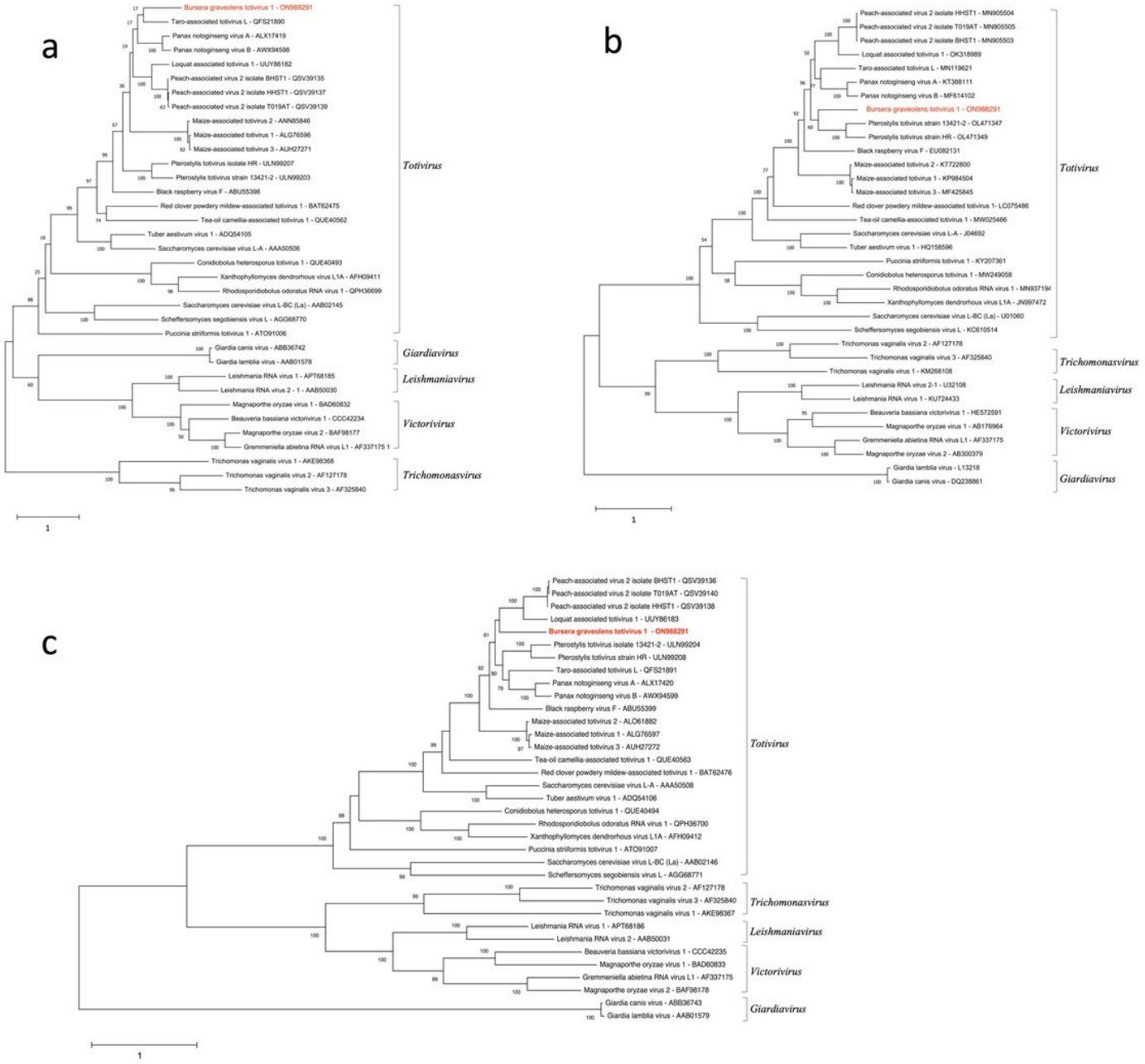

Figure 2

Maximum-likelihood phylogenetic analysis of deduced amino acid sequences of the capsid (CP) (A), CP-RNA-dependent-RNA-polymerase (RdRp) fusion protein (B), and the RdRp (C) of representative members of the family *Totiviridae*. The best-fit substitution models were (WAG+G+F), (LG+G+I+F) and (LG+G+I) for the CP, CP-RdRp, and RdRp, respectively, with 1000 bootstrap replicates. The phylogenetic tree was constructed using MEGA X. Bootstrap values are shown at the nodes. NCBI Genbank accession numbers are written next to the taxa. The new Bursera graveolens totivirus 1 is highlighted in red.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Burseratotivirus1completeON988291.txt
- OnlineResource1.docx
- OnlineResource2.docx