Evolutionary relationships and secondary structural analysis of the human filarial parasite *Onchocerca volvulus* based on 28S rRNA Sequences

Running head: Evolutionary relationships of 28S rRNA from *O. volvulus*

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Abstract

Onchocerca volvulus is the causative agent of onchocerciasis (river blindness), transmitted between hosts by blackflies of the genus *Simulium*. This study aimed to compare the partial 28S ribosomal RNA (rRNA) sequence of *O. volvulus* to determine its secondary structure and molecular phylogeny. Using PCR, an 861-bp genomic DNA fragment from *O. volvulus*, designated as *Ov*28SrRNA was successfully amplified and sequenced. The PCR products were fractionated on an agarose gel and subsequently sequenced; yielding a fragment of 861 bp. Taxonomic analysis identified 96 sequence matches within the superfamily *Filarioidea*, with 93 hits associated with the family *Onchocercidae*. A comparative search using the Rfam database revealed high similarity to known eukaryotic large subunit rRNA sequences (RF02543), corresponding to one hit for *O. volvulus*. Multiple sequence alignment of *O. volvulus* with 11 *Onchocerca* species revealed a sequence similarity range of 85.73%-92.45%. The RNAfold algorithm predicted an optimal secondary structure with a minimum free energy of -258.31 kcal/mol and an ensemble diversity of 140.91, indicating considerable structural variability. Phylogenetic analysis revealed that *O. volvulus* formed a sub-cluster with *O. lienalis* and *O. ochengi* and *O. lupi*. The lowest genetic distance (0.8%) was observed

between *O. volvulus* and the two bovine filarial parasites *O. lienalis* and *O. ochengi*, as well as *O. lupi*, a parasite of carnivores. Understanding the evolutionary relationships of *O. volvulus* may provide valuable insights into the bovine parasite-host model, which could be utilized for screening chemical compounds with potential applications against the human parasite.

Key words: 28S rRNA, filarial parasite, nematode, *Onchocerca volvulus*, secondary structure

1. Introduction

Onchocerca volvulus (Nematoda: *Filarioidea*) is a human filarial parasite responsible for onchocerciasis, commonly known as river blindness. This debilitating disease is transmitted between hosts by black flies of the genus *Simulium* and poses a significant public health challenge, causing both cutaneous and ocular manifestations that can lead to severe complications, including blindness. According to the World Health Organization (WHO), over 110 million people received specific treatment for onchocerciasis in 24 tropical countries, including regions across tropical Africa, as well as isolated endemic areas in Yemen, South America, and Central America. In 2014, large-scale treatment efforts were implemented to combat this disease (1). *Onchocerca* is one of the largest genera within the family *Onchocercidae* (Nematoda; *Spirurida*; *Filarioidea*), comprising 34 species with a worldwide distribution (2). Most members of this genus parasitize ungulates; however, two notable exceptions include *Onchocerca lupi*, which infects carnivores (3), and *O. volvulus*, the causative agent of human onchocerciasis (4). In recent years, increasing reports of zoonotic *Onchocerca* infections have drawn significant attention. Several cases have been associated with the following species: *O. dewittei japonica* (5), *O. gutturosa* in cattle (6), *O. cervicalis* in horses (7), *O. takaokai* in wild boars (8), and *O. jakutensis* in cervids (9).

Although a considerable amount of information is available regarding the life cycle of this filarial parasite, further research on closely related species is essential for identifying novel chemotherapy targets. Progress in this area is hindered by the limited availability of experimental material, either due to the parasite's complex lifecycle or the absence of reliable laboratory cultivation methods. Recent phylogenetic studies have utilized mitochondrial markers such as NADH dehydrogenase subunit 5, 16S rDNA, and 12S rDNA (10, 11). However, the application of these markers has been largely restricted to *Onchocerca* species found in Bovidae and *O. volvulus*.

The ribosomes in eukaryotic organisms consist of cytosolic particles encoded by ribosomal RNAs (rRNA) that play a critical role in the structural and functional integrity of ribosomes. They provide a detailed record of evolutionary history and serve as an essential tool for phylogenetic analysis. Ribosomal RNAs (rRNAs), in particular, have been used extensively in the phylogenetic analysis of eukaryotes, including parasitic nematodes such as *Onchocerca volvulus* (12). Each eukaryotic 80S ribosome consists of two subunits: the small 40S subunit and the large 60S subunit, which houses four rRNAs **(**28S, 18S, 5.8S, and 5S**).** Across all examined organisms, rRNA genes have been found in tandemly repeated units with a high degree of conservation. The 28S rRNA contains a pair of conserved domains that facilitate the analysis of closely related species (13). The length of these repeating units varies significantly among organisms, ranging from 44 kb in mice to 7.8 kb in soybeans (14). Within the rDNA of multiple species, repeat length variability often results from differences in the number of short repeats within the non-transcribed spacer region (15).

The objective of this study was to characterize the partial 28S rRNA sequence of *O. volvulus*, by predicting its secondary structure and comparing with the related species to determine its evolutionary conservation.

2. Materials and methods

2.1. Genomic DNA extraction

Purified *O. volvulus* worm tissue was crushed in the presence of liquid nitrogen, and then homogenized in 10 ml of RSB buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl/25 mM EDTA, SDS 1%) and incubated with DNase-free RNase (20 µg/ml) and in protease K (20 µg/ml) for 1 h at 37 °C until the material was completely dissolved. The DNA was extracted twice with an equal volume of phenol/chloroform, and the aqueous phase was then extracted with chloroform. The DNA pellet was desalted by rinsing with 70% ethanol then dissolved in TE buffer (10 mM Tris-HCl, 1 mM, EDTA, pH 7.4).

2.2. PCR amplification

Primers were designed based on the sequence data of the 28S ribosomal RNA gene from *Mansonella ozzardi* (MN432519.1) (16). The sense primer (F) was selected from the nucleotide region 3494-3520**,** while the antisense primer (R**)** was chosen from the 4382-4405 regions**.** The primer sequences were as follows: Ov28F: 5'-GTTAACGAAACTTATCGATATTCAAC) and around 4382-4405 for the antisense primers Ov28R: 5'-ATTCCTCTAATCATTCGCTTTAC). PCR amplification was performed in a reaction volume of 25 µL containing 0.1 µg genomic DNA as template, 1×PCR buffer, 1.5

mM MgCl2, 250 µM dNTPs, 0.4 µM of each primer and 0. 5U of *Taq* DNA polymerase, under the following conditions: 95°C for 3 minutes, followed by 35 cycles at 94°C for 40 seconds, 50°C for 45 seconds, 72°C for 1 minute, followed by post amplification at 72°C for 5 minutes. The amplified PCR products were electrophoresed on a 1% agarose gel and stained with DNA Safe Stain (Sinaclon, Iran) prior to detection via UV transillumination. The amplified PCR products were purified using a PCR Purification Kit (Cinnagen, Tehran, Iran) and subsequently sequenced for both strands.

2.3. Sequence analysis

Sequencing was performed using the original PCR primers**,** a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). The DNA sequences comparisons were done using Basic Local Alignment Search Tool (BLAST) (blast.ncbi.nlm.nih.gov) algorithms program in the National Center for Biotechnology Information (NCBI). All sequences were aligned using clustal_W program (17) at the European Bioinformatics Institute for multiple sequence alignments (www.ebi.ac.uk/clustalw). Similar sequences are retrieved from the NCBI database. Alignment gaps were removed for the following analyses. The phylogenetic analysis and genetic distances were performed using the neighbor-joining method (18) with 1000 replicates of bootstrapping using the MEGA11 (19). The nucleotide sequences were aligned using MUSCLE (20) and P distance method was used to estimate evolutionary distances in the trees. The fractional GC content of nucleic acid sequences were figured out using at the sequence manipulation Suit program (http://www.bioinformatics.org/SMS/). To investigate rRNA function and evolutionary relationships, the Rfam server **(**rfam.org/**)** was utilized for sequence analysis and classification. The secondary structures of rRNA were predicted using the RNAfold web server **(**rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi**)** previously described (21).

3. Results

3.1. PCR amplification and sequence analysis

To amplify the 28S rRNA of *O. volvulus*, forward and reverse primers were designed using sequence data from the corresponding gene of *M. ozzardi*, a human filarial parasite. These primers were used in a polymerase chain reaction (PCR)**,** yielding an amplified product of 861 bp (Figure 1). The nucleotide composition of the amplified sequence was as follows: 285 adenine (A, 33%), 117 cytosine (C, 14%), 172 guanine (G, 20%), and 287 thymine (T, 33%). The proportion of purine nucleotides (53%) was higher than that of pyrimidine nucleotides

(47%), with a GC content of 34%. The amplified *O. volvulus* sequence was further analyzed using the BLASTn algorithm in the NCBI GenBank database. Comparative analysis of this sequence with related sequences in 53-100% overlapping regions revealed high similarity to the majority of 28S rRNA genes from nematode parasites, with sequence identity ranging from 85.73% to 92.45% in the aligned regions. The identity percentage of the analyzed sequence was compared with the eleven available *Onchocerca* sequences in the GenBank database, including *O. lienalis* (KX853347.1), *O. ochengi* (KP760400.1), *O. lupi* (KX853349.1), *O. fasciata* (MG188680.1), *O. japonica* (KP760397.1), *O. suzukii* (KX853350.1), *O. armillata* (KX853343.1), *O. gutturosa* (KP760399.1), *O. skrjabini* (KP760401.1), *O. eberhardi* (KP760398.1) and *O. cervipedis* (KX853345.1**).** Within 53% overlapping regions**,** sequence identity ranged from 95.49% to 99.12%**.**

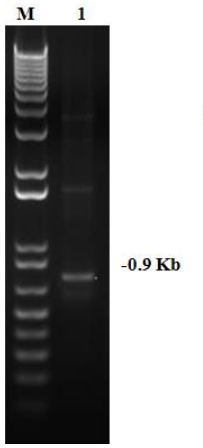


Fig. 1 PCR products of partial sequence of 28S rRNA from *O. volvulus* on 1% agarose gel. The size of DNA marker was on the right.

Taxonomic analysis of the sequence identified 96 matches within the superfamily *Filarioidea*, while 93 matches were specifically associated with the family *Onchocercidae*, highlighting its phylogenetic placement within filarial nematodes. Among these, 16 hits corresponded to species within the genus *Onchocerca*. Notably, *O. lienalis*, *O. lupi*, and *O. armillata* each had two matches, whereas the remaining *Onchocerca* species were represented by a single hit. The hits from various *Onchocerca* species further support the evolutionary conservation of this rRNA region. To explore evolutionary relationships, the *Ov*28SrRNA sequence was queried against the Rfam database. The search identified similarities with sequences annotated as Eukaryotic large subunit rRNA (accession RF02543), including one hit

(URS0000C55201_6282) corresponding to *O. volvulus*.

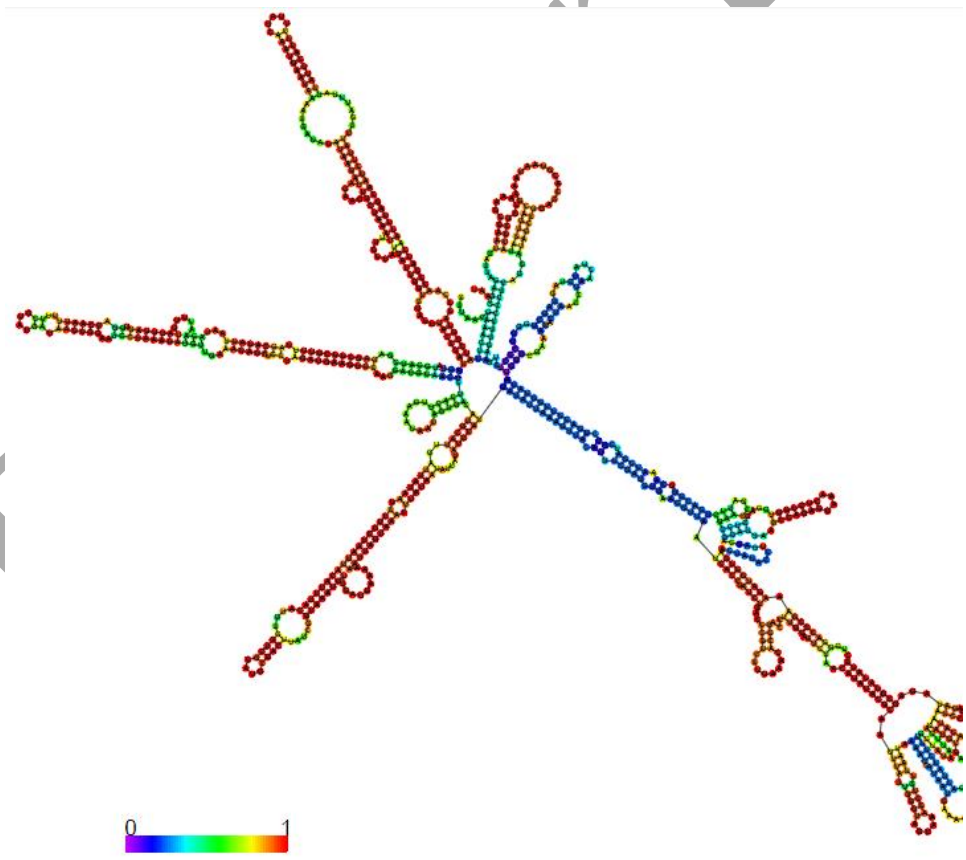
To compare the amplified nucleotide sequence of *O. volvulus*, multiple sequence alignment was performed using the Clustal_W program. Since the amplified fragment of *O. volvulus* shared 53% sequence overlap with the 3′-end of the 28S ribosomal RNA sequence from various *Onchocerca* species, only this conserved region was included in the alignment (Figure 2). When *Ov*28rRNA was aligned with *O. volvulus* (URS0000C55201_6282), *O. lienalis* (KX853347.1), *O. ochengi* (KP760400.1), *O. lupi* (KX853349.1), and *O. gutturosa* (KP760399.1), which are associated with domestic animals, only one gap was identified in *O. gutturosa* at positions 349-358, based on *Ov*28SrRNA numbering. The remaining regions were gap-free. The amplified nucleotide sequence of *Ov*28SrRNA exhibited the highest similarity (98.24%) with all *Onchocerca* species except for *O. gutturosa*, which displayed the lowest similarity (95.52%). When comparing *Ov*28SrRNA with the only available sequence of *O. volvulus* (URS0000C55201_6282), a single missing guanine (G) was observed at position 253, along with two thymine (T) substitutions for adenine (A) at positions 451-542.

Figure 2. Multiple sequence alignment of the partial *Ov*28SrRNA sequence with homologous sequences isolated from domestic animals. Identical residues are shaded in black, while conservative substitutions are highlighted in gray, indicating evolutionary conservation and sequence variability.

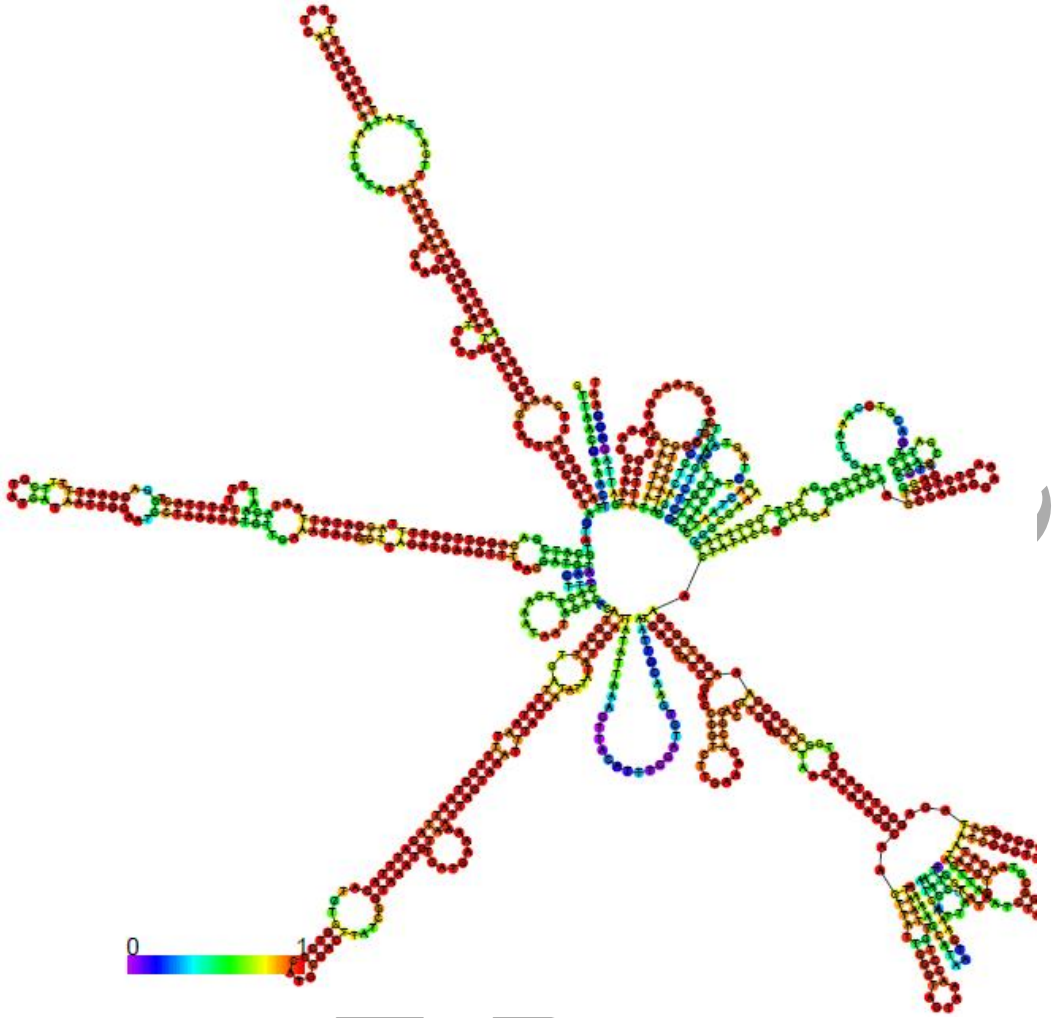
4.2. Secondary Structure Prediction of Ov28SrRNA

The sequence of *Ov*28SrRNA (Figure 3A) was analyzed for secondary structure prediction using the RNAfold program. The algorithm calculated a minimum free energy (MFE) of –258.31 kcal/mol for the thermodynamic ensemble, with an ensemble diversity of 140.91, indicating considerable structural variability. The optimal secondary structure, represented in dot-bracket notation, is shown in Figure 3B. Additionally, the optimal (Figure 3A) and centroid (Figure 3B) secondary structures provide a consensus representation, reflecting the average base-pairing probabilities across the thermodynamic ensemble. Mountain plot analysis (Figure 3C) illustrating base-pairing probabilities by comparing the optimal secondary and centroid structure to provide insights into structural stability and variability.

A)



B)



C)

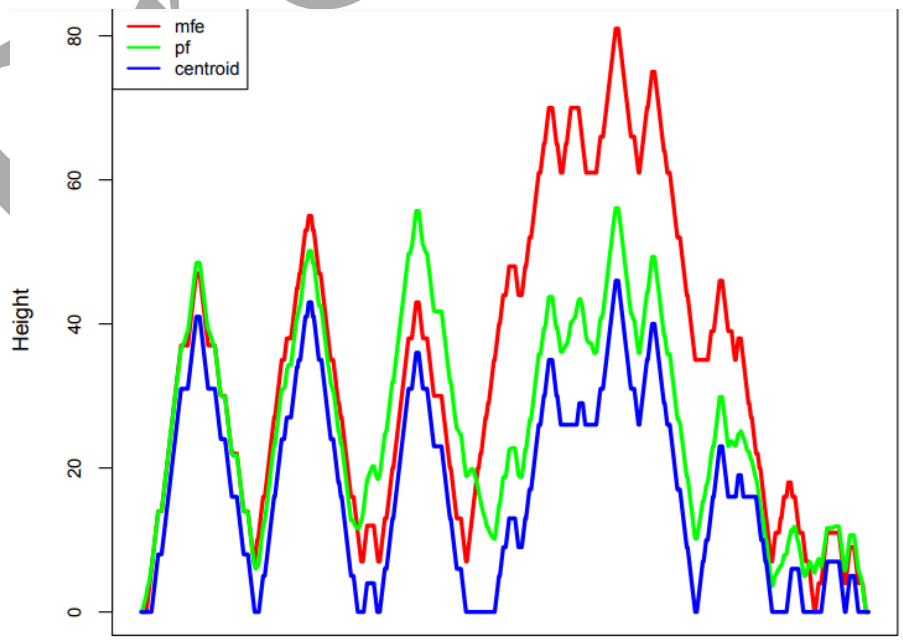


Figure 3. Predicted secondary structure of *OV*28SrRNA. (A) Optimal secondary structure prediction based on minimum free energy (MFE), encoding base-pair probabilities. (B) Centroid secondary structure prediction, encoding base-pair probabilities. (C) Mountain plot analysis generated by RNAfold, illustrating base-pairing probabilities across all possible secondary structures. The plot compares the optimal secondary structure (red line), centroid structure (green line), and pair probabilities (blue line), highlighting structural similarities and variations.

3.3 Phylogenetic analysis and genetic distances

To investigate the phylogenetic placement of the amplified *O. volvulus* sequence within the broader context of nematode 28S rRNA genes, it was compared with 12 other *Onchocerca* species, one *Loxodontofilaria* species available in GenBank, six additional filarial parasites, and one out-group. The phylogenetic tree was constructed using MEGA11 tree-drawing software from a multiple sequence alignment generated by the Clustal_W program. The neighbor-joining method was employed as the distance-based approach for tree generation. The inferred phylogenetic relationships of the amplified *O. volvulus* sequence in comparison with other *Onchocerca* species are depicted in Figure 4. The phylogenetic tree confirms that the all *Onchocerca* species, along with the single *Loxodontofilaria caprini* sequence available in GenBank, formed a major cluster. Examination of the tree reveals that all *Onchocerca* sequences predominantly group within a single cluster. Among the analyzed sequences, *Dirofilaria immitis* was identified as the closest relative to this cluster. Given that the *O. volvulus* sequence differs from *O. lienalis* and *O. ochengi*, both parasites of domestic bovids by only three nucleotides, these species were grouped together within a sub-cluster. *O. lupi*, a parasite of carnivores, was also positioned within this sub-cluster. However, *O. gutturosa* which also infects domestic animals was placed at a more distant position. In another sub-cluster**,** *O. japonica* exhibited close phylogenetic relationships with *L. caprini*, *O. suzukii*, *O. armillata*, and *O. cervipedis*. The analysis further revealed that the major *Onchocerca* cluster shares 95% sequence similarity with another distinct cluster containing various filarial genera, including *Dirofilaria, Breinlia, Dipetalonema, Loa, Pelecitus,* and *Mansonella*. Among these genera, *Dirofilaria immitis* was more closely related to the *Onchocerca* cluster than the others. *Parabronema smithii* was used as the out-group in the phylogenetic analysis.

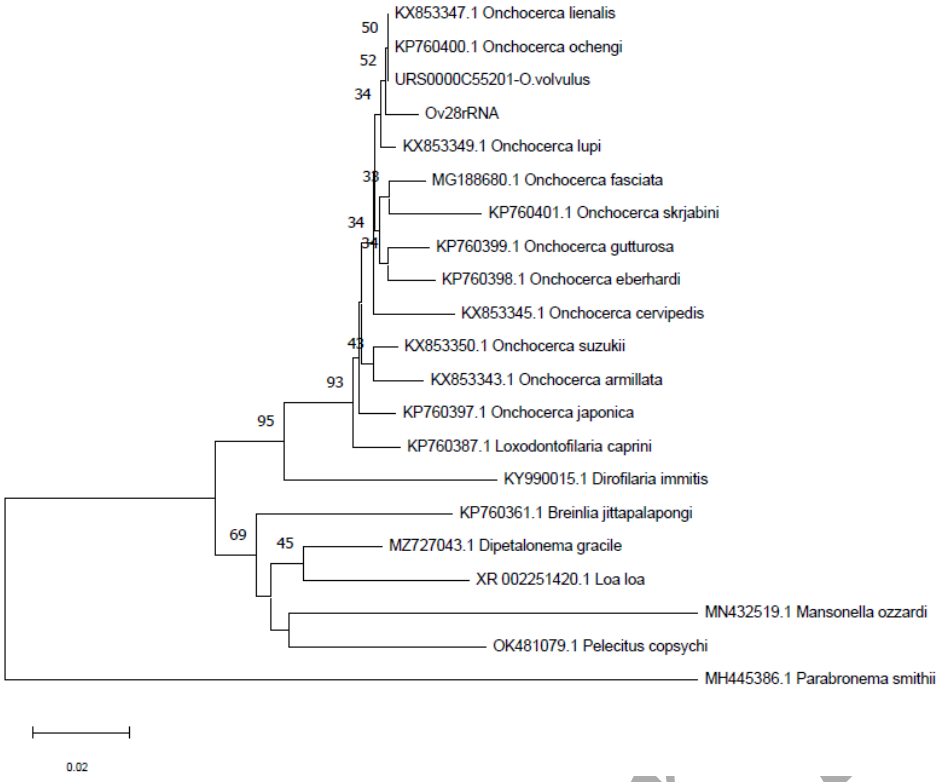


Figure 4. Neighbor-joining phylogenetic tree based on *Ov*28SrRNA sequence with the related filarial species. The numbers preceding each species indicate the GenBank accession numbers of the corresponding genes. Bootstrap values, derived from 1,000 replicates, are displayed to assess the reliability of the branching patterns. The numerical values above the lines represent the phylogenetic relationships between the groups.

The genetic pairwise distances between the amplified *Onchocerca volvulus* sequence and 12 *Onchocerca* species available in GenBank were calculated using MEGA11 software. Among these, *O. volvulus* exhibited the lowest genetic distance (0.8%) from two bovine filarial parasites, *O. lienalis* and *O. ochengi*, as well as *O. lupi*, a parasite of carnivores. In contrast, the highest genetic distance (1.5-3%) was observed between the *O. volvulus* sequence and the remaining *Onchocerca* species, as well as a single sequence from *L. caprini* (Table 1).

Table 1. Genetic pairwise distances between the amplified *Ov*28S rRNA sequence and the related species.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1-Ov28rRNA														

2-URS0000C55201	0.0													
O.volvulus	08													
3-KX853347.1 O.	0.0	0.0												
lienalis	08	00												
4-KP760400.1 O.	0.0	0.0	0.0											
ochengi	08	00	00											
5-KX853349.1 O.	0.0	0.0	0.0	0.0										
lupi	08	00	00	00										
6-MG188680.1 O.	0.0	0.0	0.0	0.0	0.0									
fasciata	20	13	13	13	13									
7-KP760397.1 O.	0.0	0.0	0.0	0.0	0.0	0.0								
japonica	15	08	08	08	08	18								
8-KX853350.1 O.	0.0	0.0	0.0	0.0	0.0	0.0	0.0							
suzukii	18	10	10	10	10	23	13							
9-KX853343.1 O.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0						
armillata	23	15	15	15	15	28	18	10						
10-KP760399.1 O.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0					
gutturosa	18	10	10	10	10	23	18	20	25					
11-KP760401.1 O.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0				
skrjabini	30	23	23	23	23	25	25	28	33	33				
12-KP760398.1 O.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
eberhardi	23	15	15	15	15	23	23	25	30	23	33			
13-KX853345.1 O.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		
cervipedis	13	13	13	13	13	25	20	23	28	23	30	28		
14-KP760387.1 L.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
caprini	20	13	13	13	13	23	15	13	18	23	35	28	25	
15-MH445386.1 P.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
smithii	19	14	14	14	14	22	09	09	17	12	27	24	19	14

4. Discussion

Onchocerca is one of the largest genera of filarial nematodes within the family *Onchocercidae*. Traditionally, species identification within this genus has relied on morphological characteristics. However, due to the close morphological similarities among

species, distinguishing them based solely on morphology remains challenging. Nematode species that cannot be reliably differentiated using morphological traits can instead be identified through molecular biomarkers, which provide a more precise and effective classification method. The use of molecular techniques is particularly valuable for resolving potential species complexes, ensuring a more accurate understanding of genetic relationships within the genus *Onchocerca*.

To amplify a partial sequence of 28S rRNA from *O. volvulus*, genetic information from *M. ozzardi*, a human filarial parasite that coexists with *O. volvulus* in South and Central America, as well as parts of the Caribbean, was utilized. Since both filarial species are transmitted in South America by infective midges from the family *Simuliidae*, sharing the same vector species, *Simulium* black flies, their biological similarities facilitated the use of *M. ozzardi* genetic data for primer design in *O. volvulus* (22).

The secondary structure prediction of the Ov28rRNA sequence, including the presence of internal loops, bulges, and base-pairing interactions**,** holds promising potential as a ligand-binding site for future small-molecule therapies**.** This predictive approach is guided by several key factors, such as the minimum free energy (MFE) of the optimal structure**,** ensemble diversity, and the thermodynamic ensemble's free energy**,** ensuring a comprehensive evaluation of structural stability and functional relevance. A highly negative value (-258.31 kcal/mol) indicates highly stable rRNA structure (-ΔG°) and strong base-pairing interactions under physiological conditions. The more negative the free energy, the more thermodynamically favorable the RNA folding is, meaning the sequence forms strong interactions and stable structures under physiological conditions. However, the fact that the free energy of the overall thermodynamic ensemble is slightly lower coupled with an ensemble diversity of 140.91, suggests a high structural variability, meaning the RNA does not settle into one dominant structure**,** instead forming many possible conformations. This could imply that *Ov*28SrRNA structure is dynamic**,** possibly involved in different interactions or conformational changes. Together, these metrics paint a picture of an RNA molecule that is thermodynamically stable overall (as inferred from the negative free energy), but does not strongly favor one dominant structure. This could suggest that the sequence is functionally flexible, potentially interacting with other molecules or undergoing structural rearrangements based on external conditions. This flexibility could play an important functional role, perhaps allowing the rRNA to interact with various ribosomal proteins or adjust to changing cellular conditions. Furthermore, rRNA structures serve as key targets for many antibiotics and small-molecule inhibitors, highlighting the critical role of secondary structure analysis in advancing

drug development and optimizing therapeutic strategies (23).

Phylogenetic analysis was conducted using 12 *Onchocerca* species alongside related nematode sequences. A detailed examination of matrix distance values within the major cluster revealed that *O. volvulus, O. lienalis, O. ochengi* and *O. lupi* are the most closely related, differing by fewer than three nucleotide changes and exhibiting the lowest genetic distance (0.8%)**.** While minor errors in sequences may arise due to non-proofreading polymerase mistakes (approximately 1×10^{-4} to 10^{-5} per base pair**),** such errors would theoretically contribute to increased genetic diversity. However, despite this possibility, the observed genetic diversity remains low, indicating that PCR-induced mutations are rare and do not significantly impact result interpretation (24). Since sequence data from this region show no major variations in nucleotide substitution rates, it provides a robust framework for evaluating relationships among closely related species. Consequently, these three organisms can be reliably distinguished by analyzing this conserved domain**.**

Multi-locus phylogenetic analyses, combined with morphological data and co-evolutionary studies of filarial nematodes with their vertebrate hosts or *Wolbachia* symbionts, suggest that the acquisition of *O. volvulus* in humans during cattle domestication may have occurred relatively recently. More specifically, a host-switching event between domestic bovines and humans appears to have taken place; supporting the hypothesis that *O. volvulus* likely diverged from an ancestral cattle parasite in Africa (11). Notably, parasites infecting domestic bovines, canids, felids, and humans appear to originate from the same lineage, indicating that domestication may have played a significant role in host-switching events. The sub-cluster formed by *O. volvulus, O. ochengi, O. lienalis,* and *O. lupi* in this study primarily consists of parasites that infect domestic animals or humans. This suggests that the domestication of cattle and dogs alongside humans likely facilitated host-switching events, contributing to speciation within this sub-cluster. Previous studies have identified several evolutionary relationships within the *Onchocerca* genus. For instance, *O. gutturosa* has been recognized as a sister species to *O. volvulus, O. ochengi,* and *O. lienalis,* while *O. volvulus* exhibits close genetic relatedness to parasites of African Bovidae, particularly *O. ochengi* (10). A separate study comparing the 5S rDNA gene of *O. lupi* with *O. gutturosa, O. cervicalis, O. ochengi,* and *O. volvulus* clearly indicated that *O. lupi* is not closely related to any of these species (2). However, in our dataset, *O. lupi, O. lienalis, O. ochengi,* and *O. volvulus* formed a distinct sub-cluster**,** suggesting that *O. lupi* is also a sister species to *O. volvulus, O. ochengi,* and *O. lienalis*. The discrepancy between prior 5S rDNA analyses and our clustering results likely stems from differences in genomic regions or methods, indicating a more complex

evolutionary relationship between *O. lupi* and its sister taxa. These findings highlight a strong evolutionary relationship among certain members of the *Onchocerca* genus, which may have practical implications for evaluating bovine parasite/host models**.** Such models could prove valuable in screening chemical compounds with potential therapeutic applications against the human parasite *O. volvulus*. The insights presented here may offer an additional objective framework for developing alternative parasite/host model systems for onchocerciasis.

In the phylogenetic tree of this study, all *Onchocerca* species clustered together with *L. caprini*, a parasite of serow (*Caprinae*) as previously suggested (1). Consistent with a prior study (8), the revision of the genus classification for this parasite was confirmed. Consequently, this species should be reassigned to the genus *Onchocerca* and designated as *O. caprini*.

Since speciation events in the genus *Onchocerca* may have occurred over several million years, relying on molecular data from a single biomarker can introduce uncertainty in phylogenetic tree construction, particularly for closely related species (25). To achieve a more comprehensive systematic analysis of this filarial nematode, further studies should incorporate additional samples, as well as nuclear and mitochondrial gene sequences.

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Authors' Contribution

Study concept and design: A.J.

Acquisition of data: A.J.

Analysis and interpretation of data: A.J.

Drafting of the manuscript: A.J.

Critical revision of the manuscript: A.J

Statistical analysis: A.J

Ethics

The author has observed all ethical points including non-plagiarism, double publication, data distortion and data manipulation in this article.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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