

Anti-oral Cancer Biological Activity Evaluation and Chloroplast Genome Analyses of *Populus euphratica*

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Abstract: In this research, the anti-cancer activity of the *Populus euphratica* extract was evaluated with Cell Counting Kit-8 (CCK-8) assay. The inhibitory activity of the *Populus euphratica* extract on the activation levels of VEGF signaling pathway in the cancer cells was measured with real time RT-PCR. Next, the high-throughput Illumina pair-end sequencing was performed to detect the chloroplast (cp) genome of *Populus euphratica* for genome evolution assessment. The CCK-8 results indicated that the extract of *Populus euphratica* exhibited the significantly suppression effect on the viability of the cancer cells, and the data of the real time RT-PCR showed the activation levels of VEGF signaling pathway in the cancer cells was also reduced obviously by the *Populus euphratica* extract. The circular cp genome of the *Populus euphratica* is 157,806 bp, encoding 131 genes, containing 8 Ribosomal RNA genes (rRNAs), 37 Transfer RNA genes (tRNAs) and 86 Protein coding genes (PCGs). And the results of the phylogenetic analysis indicated that the *Populus euphratica*. Furthermore, phylogenetic analysis revealed that *Populus euphratica* has the closest relationship with *Populus pruinosa*. In addition to *Populus pruinosa*, *Populus ilicifolia* also has closely relationship with *Populus euphratica*. These three species could be clustered on the same clade.

Key words: *Populus euphratica*, oral cancer, chloroplast genome, phylogenetic analysis

1 Introduction

Oral cancer is the malignant tumors that occur in the oral cavity, including tongue cancer, gum cancer, buccal mucosal cancer, lip cancer, and jaw cancer^{1,2)}. Oral cancer is the fourth cause of death and the number of cancer deaths among male Chinese. Smoking will greatly increase the incidence of oral cancer. The treatment of oral cancer has achieved great advances. However, up to now, only a small number of patients could be totally cured, and the final outcome of most patients is still not optimistic. The recurrence and metastasis of malignant tumors is still the biggest risk³⁾. In 1999, Tubiana reported that 45% of cancer patients could be thoroughly cured. Over the past ten years, a lot of progress has been made, and at present, nearly 50% of malignant tumor patients can survive for more than 5 years, indicating more than half are still of malignant tumor patients cannot be cured. The surgery, radiation, chemotherapy, and immunotherapy were the common therapy method for the cancer treatment, which could sig-

nificantly reduce the cancer cells. However, these treatment method could not avoid the adverse reactions and the production of cancer-resistance. Thus, new candidates for the cancer treatment needed to be developed. In the recent years, the natural products have drawn the attention of most researchers, because the excellent inhibitory activity of this natural products on the cancer treatment^{4,5)}. As reported, there were more than 50% of the anti-cancer drugs used in hospitals are derived from plant.

As a member of the *Populus* species, the *Populus euphratica* is widely exists in Mongolia, Soviet Union, Egypt, Syria, India and other places. The *Populus euphratica* of China mainly distributed in Xinjiang, the vast area between 37 degrees and 47 degrees north latitude⁶⁾. There were many reports about the multiple pharmacological activities of the *Populus euphratica*. The resin of the effect of pain relieving, which could be used for sore throat, toothache, lymph node tuberculosis, stomach and duodenal ulcer. As reported, the leaf of the *Populus euphratica* can be

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used for hypertension treatment. The inflorescence of the *Populus euphratica* was used for hemostasis⁷⁻¹⁰. While, the anti-cancer effect of the *Populus euphratica* has not been evaluated up to now. And the phylogenetic relationship between *Populus euphratica* and other *Populus* species remains unclear. Thus, in this present research, the inhibitory activity of the *Populus euphratica* extraction against the oral cancer was evaluated and the phylogenetic analysis was conducted for evolution revealing of the *Populus* species. We aimed to develop new candidates for the tumor treatment. In this present research, the biological activity evaluation revealed that the of the *Populus euphratica* was excellent on the oral cancer treatment by inhibiting the cancer cell viability, migration and invasion, and the intraspecific genetic diversity, genome evolution assessment was carried out for the reveal of relationship between *Populus euphratica* and other species by measuring the whole chloroplast genome of *Populus euphratica*.

2 Methods

2.1 Extraction

In this present research, for the extraction of mainly material in *Populus euphratica*, the soxhlet apparatus was applied. Briefly, 100 mg *Populus euphratica* fresh leaves were dissolved with 100 mL EtOH in a 50 mL flask. Next, the then the soxhlet extractor was placed onto the flask containing the extraction solvent. The flask was heated for 4-5 h, the solvent was evaporated and the desired extraction compound (white powder) was obtained¹¹.

2.2 CCK-8 assay

In this present research, the inhibitory activity of the *Populus euphratica* extract on the oral cancer treatment was evaluated with Cell Counting Kit-8 assay. This preformation was conducted strictly under the guidance of the instructions with some modifications for the viability evaluation of the oral cancer cells after treated with the *Populus euphratica* extract¹². In brief, the oral cancer cells in the logical growth phase were collected and seeded into the 96 well plates at the final density of 10^4 cells per well. Then, the cells were placed in the incubator at the condition of 37°C and 5% CO₂ for 12 h. Next, the *Populus euphratica* extract was added into the wells for treatment for 48 h with serial different dilutions (1, 2, 4, 8, 10, 20, 40 and 80 µM). Subsequently, the culture medium was discarded and the fresh medium containing 10 µL CCK-8 reagent was added. Finally, the absorbance of each well was measured with microplate reader at 450 nm. This preformation was conducted triplicate and the results were presented as mean ± SD.

2.3 Real time RT-PCR

After treated with *Populus euphratica* extract, the changes of the VEGF signaling pathway activation levels in the oral cancer cells was determined with real time RT-PCR. This experiment was performed totally in accordance with the instructions with only a little change¹³. Shortly, the oral cancer cells in the logical growth phase were collected and seeded into the 6-well plates at the density of 5×10^5 cells/well in DMEM-FBS medium. After 24 h incubation in an incubator at the 37°C, 5 % CO₂ condition, the cells were collected and the total RNA in the cells were extracted with TRIZOL reagent. The quality of the total RNA was evaluated using the OD260/OD280 ratio, and the cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cambridge, MA, USA). The PCRs were conducted using the qRT-PCR miRNA Detection Kit (Invitrogen): 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. Each experiment was performed in triplicate and the relative quantification was analyzed by the $2^{-\Delta\Delta Ct}$ method. All the results were presented as mean ± SD.

2.4 *Populus euphratica* materials and DNA isolation

Fresh leaves of *Populus euphratica* were collected from Xinjiang, China (86° 06' E, 41° 68' N), and Kunming Institute of Botany, Chinese Academy of Sciences was co-operated with our lab for further analyses¹⁴. The duplicate specimens were also saved in the herbarium of Kunming Institute of Botany (KIB) at the condition of -80°C. Ezup Plant Genomic DNA Prep Kit (Sangon Biotech, Shanghai, China)^{13, 14}. Ezup Plant Genomic DNA Prep Kit (Sangon Biotech, Shanghai, China) was used to extract with the chloroplast genomic DNA, and the chloroplast genomic DNA was used to measure the quality and quantity of the chloroplast genomic DNA.

2.5 Genome assembly and annotation

The TruSeq DNA sample preparation kits (Illumina, San Diego, CA, USA) was used to construct a paired-end library firstly. This preformation was carried out totally under the guidance of the instructions with some modifications. Then, the Illumina HiSeq 2500 platform (Illumina, San Diego, CA) of Kunming Institute of Botany (Kunming, China) was used to sequence the whole genome of *Populus euphratica* with an average read length of 150 bp pair-end. MITObim 1.8 software (Hahn, Bachmann, & Chevreux, 2013) and metaSPAdes (Nurk, Meleshko, Korobeynikov, & Pevzner, 2017) were used to assemble chloroplast genomes. The *Populus qamdoensis* (GenBank: NC040868) were chosen as the reference genome. The chloroplast genome was annotated with DOGMA software and the results were corrected with Geneious 8.0.2 (Campos *et al.*, 2016) and Sequin 15.50 (<http://www.ncbi.nlm.nih.gov/Sequin/>)¹⁵.

The parameter was set as follows: Standard Mode, "Sequence source" was set as "Plastid". Then, selecting display photosystem I, photosystem II, cytochrome b/f complex, ATP synthase, NADH dehydrogenase, RubisCO large subunit, RNA polymerase, Ribosomal proteins (SSU), ribosomal proteins (LSU), clpP, matK, other genes, hypothetical chloroplast reading frames (ycf), ORFs, transfer RNAs, ribosomal RNAs, origin of replication and polycistronic transcripts and other gene and characteristic information. Selecting "Draw GC content graph" and "Label intron-containing genes with *". The export file format was set to "PDF". Finally, submit the data and parameter settings for calculation, and finally generate a gene map of the chloroplast genome of the *Populus euphratica*. Complete cp genome includes protein coding genes, tRNAs and rRNAs, the annotations of the cp genome was carried out using Dual Organellar GenoMe Annotator (DOGMA) according to default values as described by Wyman *et al.* The tRNAscan-SE 1.23 program (<http://lowelab.ucsc.edu/tRNAscan-SE/>, Schattner *et al.* 2005) was used to verify the tRNA genes and the OGDRAW v1.2 (Lohse *et al.* 2007) was applied for the circular gene map draft. The data that support the findings of this study are openly available in GenBank at <https://www.ncbi.nlm.nih.gov>, MT593375.1.

2.6 Molecular phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible (GTR) model^{16, 17}. The tree with the highest log likelihood (-286231.95) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the BioNJ method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.00% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences. There was a total of 169844 positions in the final dataset. Evolutionary analyses were conducted in MEGA7¹⁸.

3 Results

3.1 Excellent inhibitory activity of the *Populus euphratica* extract on oral cancer cells

The widely biological activity of the *Populus euphratica* extract, such as anti-inflammatory and anticoagulant activity has drawn the attention of most researchers. However, the application values of the *Populus euphratica* extract on cancer treatment was still need to be explored. Thus, in this present research, the CCK-8 assay was firstly performed to evaluate the anti-viability of the *Populus eu-*

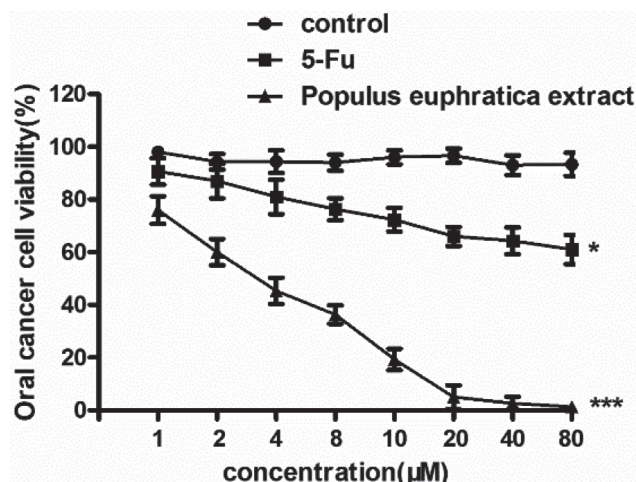


Fig. 1 *Populus euphratica* extract significantly reduced oral cancer cells viability. The oral cancer cells in the logical growth were collected and seeded into the 96 well plates at the density of 10^5 cells/well. Next, the *Populus euphratica* extract was added into the wells for 48 h incubation. The CCK-8 assay was conducted and the viability of the cancer cells was determined. * means $p < 0.05$ and *** means $p < 0.005$.

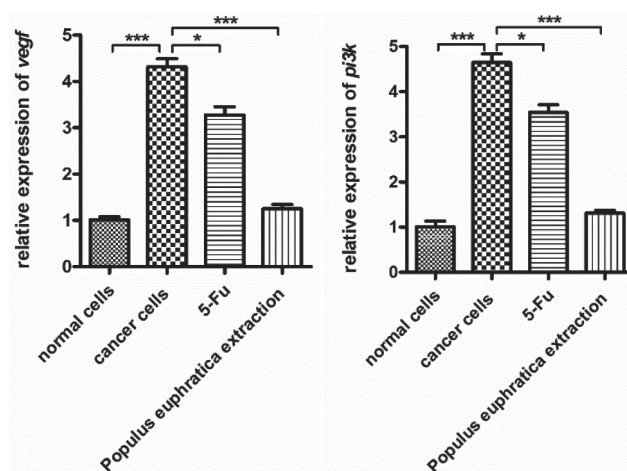


Fig. 2 *Populus euphratica* extract obviously inhibited activation levels of VEGF signaling pathway of the oral cancer cells. The oral cancer cells in the logical growth were collected and seeded into the 6 well plates at the density of 10^5 cells/well. Next, the *Populus euphratica* extract was added into the wells for indicated treatment. The real time RT-PCR was conducted and the activation levels of VEGF signaling pathway was measured. * means $p < 0.05$ and *** means $p < 0.005$.

phratica extract on the oral cancer cells. The results in Figure 1 showed that the viability of the oral cancer cells after *Populus euphratica* extract treatment was signifi-

Table 1 Genes contained in *Populus euphratica* chloroplast genome (131 genes).

Category	Group of gene	Name of gene			
Self-replication	Ribosomal RNA genes	<i>rrn4.5^a</i>	<i>rrn5^a</i>	<i>rrn16^a</i>	<i>rrn23^a</i>
	Transfer RNA gene	<i>trnH</i> -GUG	<i>trnK</i> -UUU	<i>trnL</i> -UAG ^a	<i>trnS</i> -GCU
		<i>trnG</i> -UCC*	<i>trnR</i> -UCU	<i>trnC</i> -GCA	<i>trnD</i> -GUC
		<i>trnY</i> -GUA	<i>trnE</i> -UUC	<i>trnT</i> -GGU	<i>trnS</i> -UGA
		<i>trnG</i> -GCC*	<i>trnI</i> ^f M-CAU	<i>trnS</i> -GGA	<i>trnT</i> -UGU
		<i>trnL</i> -UAA*	<i>trnF</i> -GAA	<i>trnV</i> -UAC	<i>trnM</i> -CAU
		<i>trnW</i> -CCA	<i>trnP</i> -UGG	<i>trnL</i> -CAU ^a	<i>trnL</i> -CAA ^a
		<i>trnV</i> -GAC ^a	<i>trnI</i> -GAU ^a *	<i>trnA</i> -UGC ^a *	<i>trnR</i> -ACG ^a
		<i>trnN</i> -GUU ^a			
	Small subunit of ribosome	<i>rps2</i>	<i>rps14</i>	<i>rps4</i>	<i>rps18</i>
		<i>rps12^{a,b}**</i>	<i>rps11</i>	<i>rps8</i>	<i>rps3</i>
		<i>rps19^a</i>	<i>rps7^a</i>	<i>rps15</i>	
	Large subunit of ribosome	<i>rpl33</i>	<i>rpl20</i>	<i>rpl36</i>	<i>rpl14</i>
		<i>rpl16</i>	<i>rpl22</i>	<i>rpl2^a*</i>	<i>rpl23^a</i>
	RNA polymerase subunits	<i>rpoC2</i>	<i>rpoC1*</i>	<i>rpoB</i>	<i>rpoA</i>
Photosynthesis	Subunits of photosystem I	<i>psaB</i>	<i>psaA</i>	<i>psaI</i>	<i>psaJ</i>
		<i>psaC</i>	<i>ycf3**</i>		
	Subunits of photosystem II	<i>psbA</i>	<i>psbK</i>	<i>psbI</i>	<i>psbM</i>
		<i>psbD</i>	<i>psbC</i>	<i>psbZ</i>	<i>psbJ</i>
		<i>psbL</i>	<i>psbF</i>	<i>psbE</i>	<i>psbB</i>
		<i>psbT</i>	<i>psbN</i>	<i>psbH</i>	
	Subunits of cytochrome	<i>petN</i>	<i>petA</i>	<i>petL</i>	<i>petG</i>
		<i>petB*</i>	<i>petD</i>		
	Subunits of ATP synthase	<i>atpA</i>	<i>atpF*</i>	<i>atpH</i>	<i>atpI</i>
		<i>atpE</i>	<i>atpB</i>		
	Large subunit of Rubisco	<i>rbcL</i>			
	Subunits of NADH	<i>ndhC</i>	<i>ndhB^a*</i>	<i>ndhD</i>	<i>ndhA*</i>
	Dehydrogenase	<i>ndhJ</i>	<i>ndhK</i>	<i>ndhF</i>	<i>ndhE</i>
		<i>ndhG</i>	<i>ndhI</i>	<i>ndhH</i>	
Other genes	Maturase	<i>matK</i>			
	Envelope membrane protein	<i>cemA</i>			
	Subunits of acetyl-CoA	<i>accD</i>			
	C-type cytochrome synthesis gene	<i>ccsA</i>			
	Protease	<i>clpP**</i>			
Unknown function	Conserved open reading frames	<i>ycf4</i>	<i>ycf2^a</i>	<i>ycf15^a</i>	<i>ycf1^a</i>

Note: ^a Two gene copies in IRs; ^b gene divided into two independent transcription units; one and two asterisks indicate one- and two-intron containing genes, respectively.

cantly reduced, which is significantly different from the control group, with $p < 0.005$. The inhibition of the *Populus euphratica* extract was even much better than the positive anti-cancer drug 5-Fu. This experiment revealed that the *Populus euphratica* extract exhibited pro-

tentional application values against the oral cancer.

3.2 Strong inhibition effect of the *Populus euphratica* extract on VEGF signaling pathway in the cancer cells

As we have showed in Fig. 1, the *Populus euphratica*

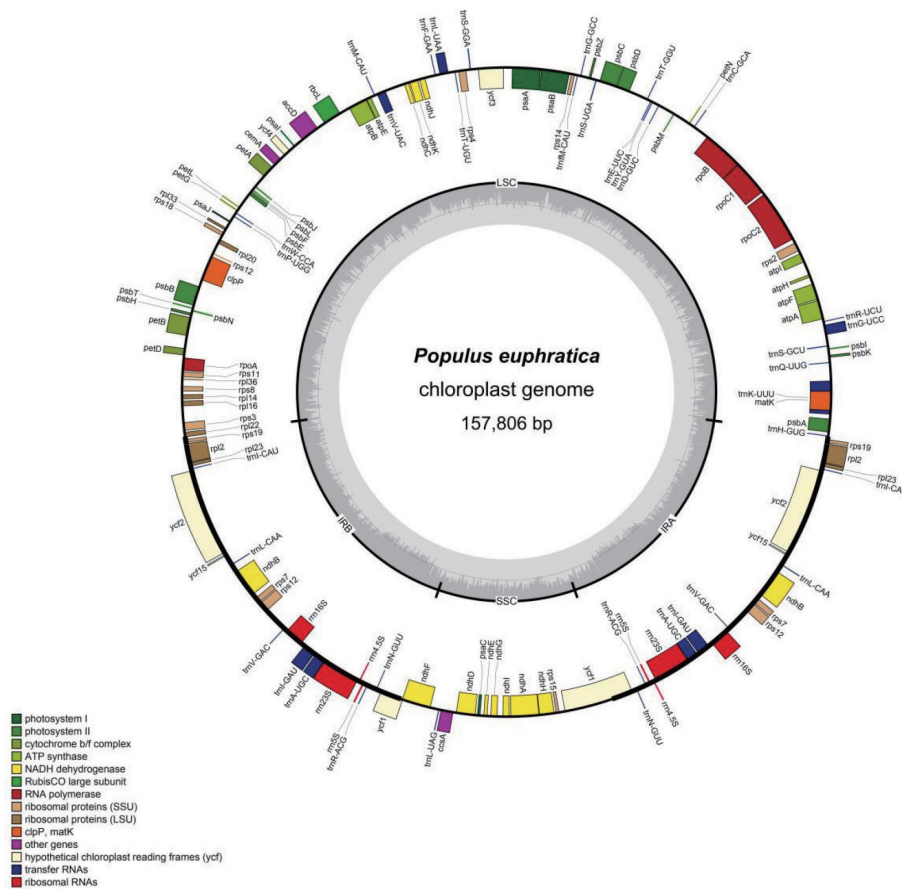


Fig. 3 Gene map of the *Populus euphratica* chloroplast genome. The outside genes are counterclockwise transcribed, the inside genes are clockwise transcribed. The different functional genes are indicated with the same color bars. Black lines presented the SSC small single copy, LSC large single copy and IR inverted repeats.

extract has excellent inhibitory activity on the viability of the oral cancer cells. In addition to the character of abnormal proliferation, the activation levels of VEGF signaling pathway in the cancer cells was also need to be paid attention, which is important for the cancer cell proliferation. As the results showed in **Fig. 2**, we can see that there was a higher activation level of the oral cancer cells compared with the normal cells. However, after the treatment of the *Populus euphratica* extract, the activation levels of VEGF signaling pathway in the cancer cells was reduced obviously.

3.3 Chloroplast genome features

After the chloroplast genome sequence of *Populus euphratica* and the annotation analysis, we can see in **Table 1**, there were four categories and totally 131 genes in the chloroplast genome (Self-replicating genes, Photosynthesis genes, Other functional genes and Unknown function genes). In the Self-replicating genes, there were total 8 Ribosomal RNA genes (duplicated *rrn4.5*, *rrn5*, *rrn16*, and *rrn23*), 37 transfer RNA genes (tRNAs), 14 Small subunit of ribosome genes, 10 Small subunit of ribosome genes, 4

RNA polymerase subunits. In the Photosynthesis genes, there are 21 Subunits of photosystem I and Subunits of photosystem II genes, 6 Subunits of ATP synthase genes, 1 Large subunit of Rubisco gene, 12 Subunits of NADH Dehydrogenase. In addition to this, the *matK*, *cemA*, *accD*, *ccsA* and *clpP* belong to the Other genes. And unknown function genes include of 7 genes (duplicated *ycf2*, *ycf15*, *ycf1* and a *ycf4*). The repetitive genes appearing in the inverted repeat region have been marked with "a" in the upper corner of **Table 1**.

3.4 Chloroplast genome gene map

The annotated chloroplast genome of *Populus euphratica* was saved as .gb format file, and then uploaded onto OGDRAW (<https://chlorobox.mpimgolm.mpg.de/OGDRAW.html>), an online analysis tool for the chloroplast genome physical map preparation. As the results showed in **Fig. 3**, the chloroplast genome of *Populus euphratica* has a typical tetrad structure. The circular cp genome of the *Populus euphratica* is 157,806 bp, encoding 131 genes. These predicated genes contain 8 Ribosomal RNA genes (rRNAs), 37 Transfer RNA genes (tRNAs) and 86 Protein

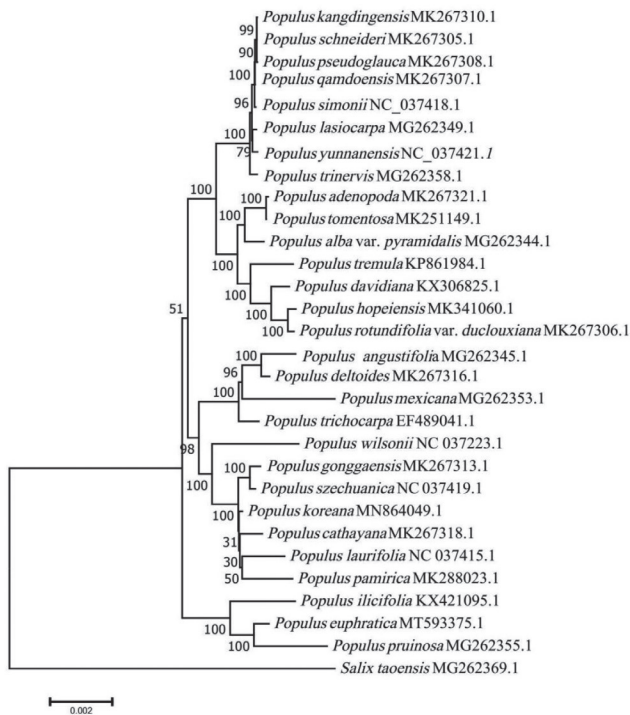


Fig. 4 Gene phylogenetic analysis of *Populus* species by Maximum Likelihood method. Phylogenetic relationships of *Populus* species based on complete chloroplast genome sequences of 29 *Populus* species and 1 outgroup species with Maximum Likelihood (ML) method.

coding genes (PCGs). In the circular cp genome of the *Populus euphratica*, there was 85,842 bp large single-copy (LSC) region, including 22 tRNAs and 59 PCGs, as well as a 16,628 bp small single-copy (SC) region, including 1 tRNA and 12 PCGs. The IRA and IRB regions have the same 20 genes with opposite directions, such as *rps19*, *rpl2*, *rpl23*, *trnI-CAU*, *ycf2*, *ycf15*, *trnL-CAA*, *ndhB*, *rps7*, *rps12*, *trnV-GAC*, *rrn16S*, *trnI-GAU*, *trnA-UGC*, *rrn23S*, *rrn4.5S*, *rrn5S*, *trnR-ACG*, *trnN-GUU* and *ycf1*.

3.5 Phylogenetic analysis

Phylogenetic analysis of *Populus* species was performed based on the whole chloroplast complete sequences of 30 species, including 29 *Populus* species and 1 *Salix* species (*Salix taoensis*) as the outgroup species (Fig. 4). As the results showed in the reconstructed phylogenetic tree, all species could be clustered into two groups, the *Populus* evolution group and the *Salix* evolutionary group. according to the evolutionary relationship, *Populus euphratica* has the closest relationship with *Populus pruinosa*, with the evolutionary similarity of 100.

4 Conclusion

In this present research, for the treatment of oral cancer, the biological activity of the *Populus euphratica* extract was evaluated, and the phylogenetic analysis of the *Populus euphratica* with other species was analyzed as well. Through the CCK-8 assay, we proved that the extract of *Populus euphratica* could significantly reduce the viability of the oral cancer cells. Besides, the activation levels of VEGF signaling pathway in the oral cancer cells was also reduced obviously by the *Populus euphratica* extract, suggesting the excellent application values of the *Populus euphratica* extract on oral cancer. Additionally, the *Populus euphratica* has a 157,806 bp length cp genome. Furthermore, phylogenetic analysis revealed that *Populus euphratica* has the closest relationship with *Populus pruinosa*. In addition to *Populus pruinosa*, *Populus ilicifolia* also has closely relationship with *Populus euphratica*. The above results suggested that these three species may also has similar anti-cancer application values, which need to be further explored.

Conflict of Interest

The authors report no conflict of interest.

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Data Availability Statement

The data that support the findings of this study are openly available in GenBank at <https://www.ncbi.nlm.nih.gov>, reference number MT593375.

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