Excellent Anti-lung Cancer Activity of *Populus nigra* and Phylogenetic Analysis

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Abstract: Lung cancer has the highest incidence rate among malignant tumors all over the world, and it is also the leading cause of death. In this present research, we aimed to evaluate the anti-cancer activity of the *Populus nigra* extract against the lung cancer and study the genome evolution of the *Populus nigra*. Firstly, the inhibitory activity of the *Populus nigra* extract on the NCI-H292 lung cancer cell viability was determined with Cell Counting Kit-8 (CCK-8) assay. The trans-well assay was conducted and the influence of the *Populus nigra* extract on the NCI-H292 lung cancer cell migration and invasion ability was determined. In addition to this, the chloroplast (cp) genome of *Populus nigra* was sequenced with high-throughput Illumina pair-end sequencing, which was a classical useful model for genome evolution assessment. The CCK-8 and trans-well assay indicated the *Populus nigra* extract exhibited excellent inhibitory activity on the NCI-H292 lung cancer cell viability, migration and invasion ability. The circular cp genome of the *Populus nigra* was 156,354 bp in size, including a large single-copy (LSC) region of 84,528 bp and a small single-copy (SC) region of 16,564 bp, which were separated by two inverted repeat (IR) regions (38,612 bp each). A total of 132 genes were predicted, including 8 ribosomal RNAs (rRNAs), 37 transfer RNAs (tRNAs), and 90 protein-coding genes (PCGs). Furthermore, phylogenetic analysis revealed that *Populus nigra* has the closest relationship with *Populus alba* var. *pyramidalis*. In addition to *Populus alba* var. *pyramidalis*, *Populus adenopoda* and *Populus tomentosa* are also has closely relationship with *Populus nigra*.

Key words: anti-lung cancer, *Populus nigra*, viability, migration, invasion, chloroplast genome, phylogenetic analysis

1 Introduction

Lung cancer is one of the malignant tumors with the fastest increase in morbidity and mortality and the greatest threat to human health and life. Therefore, the incidence and death of lung cancer have now become a major cause of death for urban residents in our country¹⁾. Traditionally, the cancer treatment commonly used in clinic were surgery, radiation, chemotherapy, and immunotherapy, which were targeted on the highly proliferation of tumor cells, but which could cause many adverse reactions and the production of cancer-resistance²⁾. Among the various effective anti-cancer drugs currently used in clinical practice, more than half of the drugs are originally derived from natural products. Statistics show that plant-derived anti-cancer drugs can account for more than 50% of the anti-cancer drugs used in hospitals, and paclitaxel ranks first among various anti-cancer drugs. *Populus nigra* is reported exhibiting multiple pharmacological activities, such as anti-inflammatory and anticoagulant activity³⁻⁶⁾. However, there is no solid record and reliable experimental data

about the anti-cancer of the *Populus nigra*. And the phylogenetic relationship between *Populus nigra* and other related members of the *Populus* species remains unclear. Thus, in this present research, the biological activity of the *Populus nigra* extraction was evaluated and the phylogenetic analysis was conducted for evolution revealing.

Populus nigra, of section *Populus*, is distributed mainly in Europe, North Africa and West Asia. And it can grow in the area from Heilongjiang and southern Inner Mongolia in the north to Guangdong and Guangxi in the south (Henan, Hubei, Hunan, Guangdong, Guangxi, Hainan) of China. *Populus nigra* has high ecological and economic value, which is usually used for wind and sand fixation, soil and water conservation. The poplar leaf of the *Populus nigra* can be used as feed for wild animals and live. The poplar bark of the *Populus nigra* contains tannin, which could be used as tanning material. Most importantly, the *Populus nigra* is reported exhibiting multiple pharmacological activities, such as anti-inflammatory and anticoagulant activity⁷⁾. However, the anti-cancer activity of the *Populus*

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nigra is still need to be explored. According to the intra-specific genetic diversity, genome evolution assessment was carried out to reveal the evolutionary relationship of the *Populus nigra* with other species^{8,9}. In this present research, the whole chloroplast genome of *Populus nigra* was constructed for population genetics studies. Above all, the *Populus nigra* extract provide a new choice for the lung cancer treatment by inhibiting the cancer cell viability, migration and invasion.

2 Methods

2.1 Extraction

In this study, soxhlet apparatus was used for the extraction of *Populus nigra*¹⁰. Typically, fresh leaves of *Populus nigra* (100 mg) was dissolved in EtOH (100 mL) in a 50 mL flask, then the soxhlet extractor was placed onto the flask containing the extraction solvent. After heating the flask for 4-5 h, the desired extraction compound was obtained via evaporation of the solvents by the rotatory evaporator as white color powder.

2.2 CCK-8 assay

The Cell Counting Kit-8 was conducted firstly in this present research to evaluate the inhibitory activity of the *Populus nigra* extract on the NCI-H292 lung cancer cell viability. This preformation was finished totally under the guidance of the instructions with some modifications^{11,12}. In brief, the NCI-H292 lung cancer cells in the logical growth phase were collected and then seeded into the 96 well plates at the final density of 10^5 cell per well. After 12 h incubation in an incubator at the condition of 37°C, 5% CO₂, the *Populus nigra* extract was added into the wells for another 12 h incubation. Then, the culture medium was discarded and the fresh medium containing the CCK-8 reagent was added. Finally, the absorbance of each well was measured with microplate reader at 450 nm. This preformation was conducted at least three times and the results were presented as mean $\pm$ SD.

2.3 Trans-well assay

The trans-well assay was conducted in this present research to evaluate the influence of the *Populus nigra* extract on the migration and invasion ability of the NCI-H292 lung cancer cells. This conduction was finished strictly in accordance with the protocols with only little change¹³. Briefly, the 24-well trans-well chambers (8 μ m pore size, 6.5 mm diameter; Corning, NY, USA) were pre-coated with or without of Matrigel matrix (50 μ L/well, 200 mg/mL; BD Biosciences, Franklin Lakes, NJ, USA), respectively for the migration and invasion assays. Then, the chambers were placed into the 24-well plates, and the cancer cells in the logical growth phase were collected and

seeded into the upper chamber at the density of 5×10^4 cells per well, cultured with free-FBS medium. After 24 h incubation in an incubator at the 37°C, 5% CO₂ condition, the cells remaining on the upper sides of the membranes were carefully wiped off, and the cells on the lower side of the membranes were labeled with 0.5% crystal violet. The cell numbers on the lower surface of the membrane were quantified in six randomly independent fields under a microscope.

2.4 Plant materials and DNA isolation

Fresh leaves of *Populus nigra* were collected from Xinjiang, China (86° 06' E, 41° 68' N), and further analyses was conducted in the Kunming Institute of Botany, Chinese Academy of Sciences. The duplicate specimens were saved in the herbarium of Kunming Institute of Botany (KIB) at -80°C condition. The chloroplast genomic DNA was extracted from 25 mg silica-gel-dried leaves with Ezup Plant Genomic DNA Prep Kit (Sangon Biotech, Shanghai, China)^{14,15}. The quality and quantity of the chloroplast genomic DNA was measured with Agarose gel electrophoresis.

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 modifications¹⁶⁻¹⁸. 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 nigra* 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/>).

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 nigra*. Complete cp genome includes protein coding genes, tRNAs and rRNAs, the an-

notations 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>, NO. MT593372.

2.6 Comparative and phylogenetic analysis

The phylogenetic analysis was conducted based on the General Time Reversible model with Maximum Likelihood (ML) method¹⁹⁾. The tree with the highest log likelihood (-297751.66) 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 automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 35 nucleotide sequences. There was a total of 169964 positions in the final dataset. The ML phylogenetic analysis were conducted with MEGA v7.0.26 generating 1000 bootstrap replicates to determine measures of nodal support with each run initiating from a random starting tree²⁰⁾.

3 Results

3.1 *Populus nigra* extract significantly reduce the viability of the cancer cells

As the excellent pharmacological activities of the *Populus nigra* extract, such as anti-inflammatory and anticoagulant activity, which application values on the lung cancer treatment was firstly explored. Thus, the CCK-8 assay was conducted in this research to assess the inhibitory activity of the *Populus nigra* extract on the NCI-H292 lung cancer cell viability. As the results showed in Fig. 1, we can see that the compared with the control group, the *Populus nigra* extract could significantly reduce the viability of the NCI-H292 lung cancer cells with $p < 0.005$, which is even better than the positive anti-cancer drug 5-Fu. This result suggested the *Populus nigra* extract has the excellent clinical application values for the cancer therapy.

3.2 *Populus nigra* extract obviously inhibit the migration and invasion ability of the cancer cells

In the above experiment, we have proved that the *Populus nigra* extract could significantly reduce the NCI-

Fig. 1 Significantly reduced viability of the cancer cells after *Populus nigra* extract treatment. The cancer cells in the logical growth were collected and seeded into the 96 well plates at the density of 10^5 cells/well. Then the *Populus nigra* extract was used for indicated treatment. The viability of the cancer cells was determined with CCK-8 assay. * means $p < 0.05$, *** means $p < 0.005$.

Fig. 2 Obviously inhibited migration and invasion ability of the NCI-H292 lung cancer cells after *Populus nigra* extract treatment. The cancer cells in the logical growth phase were collected and seeded into the well plates, then treated with *Populus nigra* extract. The numbers of migrated and invaded cells were counted. * means $p < 0.05$, *** means $p < 0.005$.

H292 lung cancer cell viability. However, whether the *Populus nigra* extract could also inhibit the NCI-H292 lung cancer cell migration and invasion ability was still need to be explored. So, the trans-well assay was conducted for this assessment. The results in Fig. 2 showed that the migration and invasion ability of the NCI-H292 lung cancer cell was much stronger than that of the normal human cells, with $p < 0.005$, while after the *Populus nigra* extract exposure, the NCI-H292 lung cancer cell migration

and invasion ability was obviously reduced.

3.3 Chloroplast genome features

According to the annotation analysis of the chloroplast genome of *Populus nigra*, there are four categories of 132 genes in the chloroplast genome, which can be divided into self-replicating genes, photosynthesis genes, other functional genes and unknown function genes. The Self-repli-

cating genes consisted of 4 duplicated Ribosomal RNA genes (*rrn4.5*, *rrn5*, *rrn16*, and *rrn23*), 37 transfer RNA genes, 14 Small subunit of ribosome genes, 10 Large subunit of ribosome genes, 4 RNA polymerase subunits genes. The Photosynthesis genes comprised of 21 genes in Subunits of photosystem I and Subunits of photosystem II, 6 genes in Subunits of cytochrome, 1 gene in Rubisco large subunit, 12 genes coding Subunits of NADH Dehydroge-

Table 1 Genes contained in *Populus nigra* chloroplast genome (133 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>trnM</i> -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>trnI</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	Translational initiation	<i>infA</i>			
	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.

nase. There are 6 genes *infA*, *matK*, *cemA*, *accD*, *ccsA*, *clpP* belong to the Other functional genes and 7 genes *ycf4*, *ycf2^a*, *ycf15^a*, *ycf1^a* belonging Unknown function genes. The repetitive genes appearing in the inverted repeat region have been marked with "a" in the upper corner of Table 1.

3.4 Gene map of chloroplast genome

The annotated chloroplast genome was saved as .gb format file, which was then uploaded onto OGDRAW (<https://chlorobox.mpimp.golm.mpg.de/OGDRAW.html>), an online analysis tool for the chloroplast genome physical map preparation. The chloroplast genome of *Populus nigra* has a typical tetrad structure (Fig. 3). The circular cp genome of the *Populus nigra* was 156,354 bp in size, including a large single-copy (LSC) region of 84,528 bp and a small single-copy (SC) region of 16,564 bp, which were separated by two inverted repeat (IR) regions (38,612 bp each). A total of 132 genes were predicted, including 8 ribosomal RNAs (rRNAs), 37 transfer RNAs (tRNAs), and 90 protein-coding genes (PCGs). The LSC consisted of 81 genes and

the SSC comprised of 13 genes. 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 was performed according to the whole chloroplast complete sequences of 35 species, including 31 *Populus* and 4 *Salix* species (*Salix matsudana* f. *tortuosa*, *Salix tetrasperma*, *Salix oreinoma* and *Salix taoensis*) as outgroup species (Fig. 4). The reconstructed phylogenetic tree clustered all the species into two groups. The four *Salix* species were clustered into the same evolutionary group, while all other *Populus* species were clustered into another major evolution group. The *Populus nigra* has the closest relationship with *Populus alba* var. *pyramidalis*. In addition to *Populus alba* var. *pyramidalis*, *Populus adenopoda* and *Populus tomentosa* are also has closely relationship with *Populus nigra*.

Fig. 3 Gene map of the *Populus nigra* chloroplast genome. Genes lying outside of the circle are transcribed counterclockwise, and the genes inside the circle are transcribed clockwise. The genes belong to different functional groups are indicated with colored bars. The SSC small single copy, LSC large single copy and IR inverted repeat are indicated are indicated with black lines.

Fig. 4 Gene phylogenetic analysis by Maximum Likelihood method. Phylogenetic relationships based on complete chloroplast genome sequences of 31 *Populus* species and 4 outgroup species with Maximum Likelihood (ML) method.

4 Conclusion

In this present research, the anti-cancer activity of the *Populus nigra* extract was evaluated and its phylogenetic analysis was conducted for evolution revealing as well. The results of the CCK-8 showed that the *Populus nigra* extract could significantly reduce the viability, migration and invasion ability of the NCI-H292 lung cancer cells, suggesting the excellent application prospect the *Populus nigra*. The length of *Populus nigra* cp genome reported in this present research is 156,354 bp, which is 392 bp similar than that of closely related species *Populus adenopoda* 156,746 bp²¹. The *Populus* species also have and similar gene orders, gene numbers and GC contents. The *Populus nigra* cp genome is AT-rich (63.23%), which is consistent with other species from *Populus* family, for example, *Populus adenopoda* (63.21%), *Populus alba* (63.26%)²², *Populus alba* var. *pyramidalis* (63.21%) and *Populus tremula* var. *davidiana* (63.21%)²¹. This evolutionary relationship suggesting the other related species may also has the anti-lung cancer activity as above

described. Through this research, we proved that the *Populus nigra* extract provide a new choice for the lung cancer treatment by inhibiting the cancer cell viability, migration and invasion. And the closely related species also have the same anti-cancer activity, which also need to be explored.

Author Contributions

Jun Ma, Yang Gao, Tao Jiang and Feng Tian contributed equally to this research. Tao Jiang and Feng Tian designed this research together; Jun Ma finished the biological evaluation of the *Populus nigra* extract; Yang Gao finished the circular cp genome sequence of the *Populus nigra* and related evolutionary relationship analysis.

Supporting Information

The data that support the findings of this study are openly available in GenBank at <https://www.ncbi.nlm.nih.gov>, reference number MT593372.

References

- 1) Torre, L.A.; Siegel, R.L.; Ward, E.M.; Jemal, A. Global cancer incidence and mortality rates and trends-An update. *Cancer Epidemiol. Biomarkers Prev.* **25**, 16-27 (2016).
- 2) Wang, J.J.; Lei, K.F.; Han, F. Tumor microenvironment: recent advances in various cancer treatments. *Eur. Rev. Med. Pharmacol. Sci.* **22**, 3855-3864 (2018).
- 3) Ni, H.; Muhammad, I.; Li, J.; Wang, B.; Sheng, Z. *In vitro* and *in vivo* antioxidant activities of the flavonoid-rich extract from *Flos populus*. *Pak. J. Pharm. Sci.* **32**, 2553-2560 (2019).
- 4) Tehrani, M.; Sankian, M.; Assarehzadegan, M.A.; Falak, R.; Noorbakhsh, R. *et al.* Identification of a new allergen from *Amaranthus retroflexus* pollen, Amar2. *Allergol. Int.* **60**, 309-316 (2011).
- 5) Kis, B.; Avram, S.; Pavel, I.Z.; Lombrea, A.; Buda, V. *et al.* Recent advances regarding the phytochemical and therapeutic uses of *Populus nigra* L. *Buds. Plants (Basel)* **9**, 1464 (2020).
- 6) Park, J.H.; Lee, T.K.; Ahn, J.H.; Shin, B.N.; Cho, J.H. *et al.* Pre-treated *Populus tomentiglandulosa* extract inhibits neuronal loss and alleviates gliosis in the gerbil hippocampal CA1 area induced by transient global cerebral ischemia. *Anat. Cell Biol.* **50**, 284-292 (2017).
- 7) Zhao, H.; Jiang, J.; Li, K.; Liu, G. *Populus simonii* × *Populus nigra* WRKY70 is involved in salt stress and leaf blight disease responses. *Tree Physiol.* **37**, 827-844 (2017).
- 8) Wu, H.; Yao, D.; Chen, Y.; Yang, W.; Zhao, W.; Gao, H.; Tong, C. *De novo* genome assembly of *Populus simonii* further supports that *Populus simonii* and *Populus trichocarpa* belong to different sections. *G3 (Bethesda)* **10**, 455-466 (2020).
- 9) Chu, Y.; Huang, Q.; Zhang, B.; Ding, C.; Su, X. Expression and molecular evolution of two DREB1 genes in black poplar (*Populus nigra*). *PLoS One* **9**, e98334 (2014).
- 10) Kuš, P.; Jerković, I.; Jakovljević, M.; Jokić, S. Extraction of bioactive phenolics from black poplar (*Populus nigra* L.) buds by supercritical CO₂ and its optimization by response surface methodology. *J. Pharm. Biomed. Anal.* **152**, 128-136 (2018).
- 11) Zhang, C.; Wang, W.; Lin, J.; Xiao, J.; Tian, Y. lncRNA CCAT1 promotes bladder cancer cell proliferation, migration and invasion. *Int. Braz. J. Urol.* **45**, 549-559 (2019).
- 12) Zhao, B.; Song, X.; Guan, H. CircACAP2 promotes breast cancer proliferation and metastasis by targeting miR-29a/b-3p-COL5A1 axis. *Life Sci.* **244**, 117179 (2020).
- 13) Ren, S.; Liu, J.; Feng, Y.; Li, Z.; He, L. *et al.* Knock-down of circDENND4C inhibits glycolysis, migration and invasion by up-regulating miR-200b/c in breast cancer under hypoxia. *J. Exp. Clin. Cancer Res.* **38**, 388 (2019).
- 14) Liu, D.; Cui, Y.; Li, S.; Bai, G.; Li, Q. *et al.* A new chloroplast DNA extraction protocol significantly improves the chloroplast genome sequence quality of foxtail millet (*Setaria italica* (L.) P. Beauv.). *Sci. Rep.* **9**, 16227 (2019).
- 15) Satjarak, A.; Graham, L.E. Comparative DNA sequence analyses of *Pyramimonas parkeae* (Prasinophyceae) chloroplast genomes. *J. Phycol.* **53**, 415-424 (2017).
- 16) Armstrong, J.; Fiddes, I.T.; Diekhans, M.; Paten, B. Whole-genome alignment and comparative annotation. *Annu. Rev. Anim. Biosci.* **7**, 41-64 (2019).
- 17) Simão, F.A.; Waterhouse, R.M.; Ioannidis, P.; Kriventseva, E.V.; Zdobnov, E.M. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* **31**, 3210-3212 (2015).
- 18) Kim, B.M.; Lee, Y.J.; Kim, J.H.; Kim, J.H.; Kang, S. *et al.* The genome assembly and annotation of the southern elephant seal *Mirounga leonina*. *Genes (Basel)* **11**, 160 (2020).
- 19) Nei, M.; Kumar, S. *Molecular evolution and phylogenetics*. Oxford University Press, New York (2000).
- 20) Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**, 1870-1874 (2016).
- 21) Zong, D.; Gan, P.; Zhou, A.; Li, J.; Xie, Z.; Duan, A.; He, C. Comparative analysis of the complete chloroplast genomes of seven *Populus* species: Insights into alternative female parents of *Populus tomentosa*. *PLoS One* **14**, e0218455 (2019).
- 22) Xu, C.; Dong, W.; Li, W.; Lu, Y.; Xie, X. *et al.* Comparative analysis of six *Lagerstroemia* complete chloroplast genomes. *Front Plant Sci.* **8**, 15 (2017).

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