



Full Length Article

Study of Kin Recognition and Physiological Strategies of Adjacent Black Pines (*Pinus thunbergii*)

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Abstract

Kin recognition of plants refers to the phenomenon, in which plants can adjust their own growth and ecological strategies to promote survival and reproduction of relatives by recognizing the relationship between neighboring individuals and them. At present, there is no study to confirm the existence of kin recognition, especially in woody plants. In this study, black pine (*Pinus thunbergii* Parl.), a typical gymnosperm and one of the important afforestation tree species, was used to construct the genetic distance through gene sequencing analysis in order to distinguish the close and distant relatives. Furthermore, chlorophyll a and b was measured calorimetrically, which represents the primary production capacity of plants and phenolic acids (ferulic acid, vanillic acid and syringic acid) was measured by high-performance liquid chromatography, which are typical plant secondary metabolites, attempting to reveal the presence of kin recognition in gymnosperms. Results showed that compared to distant relatives, the contents of chlorophyll a and b, and phenolic acids were significantly higher in the pine needles of close relatives. This study revealed that under natural conditions, *P. thunbergii* plants can recognize the close relatives of neighboring strains and adjust physiological strategies accordingly, showing the differences in primary production capacity and secondary metabolite distribution. To the best of our knowledge, this is the first study to discover and confirm the presence of kin recognition in black pine. In addition, this is second reported case of gymnosperm in the world. This study provides a new explanation for the population adaptation and niche of *P. thunbergii* in northern China, that is, *P. thunbergii* is a kinship-promoting type. © 2020 Friends Science Publishers

Keywords: Kin recognition; Black pine; Chlorophyll; Ecological niche; Kinship-promoting type

Introduction

Positive (reciprocal) and negative interactions (competition) between plants (especially neighboring woods) are important processes that affect community structure, species diversity and succession dynamics (Brooker *et al.*, 2008; Dudley *et al.*, 2013; Wright *et al.*, 2014). Some plants are more likely to be adjacent to close relatives due to inbreeding, seed transmission, vegetative reproduction or artificial selection (Lin *et al.*, 2015). According to niche theory, there is a large overlap in the niche of closely related plants, which generates intense competition (Silvertown, 2004). However, there is little evidence to support that the competition between relatives is greater than that between unrelated ones (Cheplick, 1992). Conversely, several studies have found that when plants are adjacent to their close relatives, the growth is better with increase in their strength (Ninkovic, 2003; Bhatt *et al.*, 2011; File *et al.*, 2012). Some researchers reported that individuals with high genetic similarity could reduce the consumption of resources through mutual cooperation rather than competition, thus

directing more resources in breeding and plant strength (Axelrod and Hamilton, 1981; Chen and Li, 2015).

Currently, individual kin selection behavior has been reported in animal and microbial communities, but there is ambiguity on its existence in plant communities. Previous studies have reported kin recognition in at least 20 plant species, including *Hordeum vulgare* (Ninkovic, 2003), *Cakile edentula* (Bhatt *et al.*, 2011), ragweed (*Ambrosia artemisiifolia*) (File *et al.*, 2012), *Lupinus angustifolius* (Milla *et al.*, 2012), *Arabidopsis thaliana* (Caffaro *et al.*, 2013) and rice (*Oryza sativa*) (Fang *et al.*, 2013). Nevertheless, some studies have shown negative results.

From the perspective of plant taxonomy, these more than 20 species come from 11 families and 21 genera, and the majority belongs to angiosperms (Lin *et al.*, 2015) while *Larix gmelinii* is the only gymnosperm reported (Hussain *et al.*, 2019). Except for *L. gmelinii* and *Artemisia tridentate* other studied plants were all herbaceous. Most studies focused on the indicators of the differences in phenotypes when the plant of interest co-existed with different relatives, while only few studies

investigated primary production capacity and secondary metabolites. *Pinus thunbergii*, *P. tabuliformis* and *P. koraiensis* are the main afforestation species in northern China (Zhang et al., 2018). It has been found that the population of *P. thunbergii* in the middle-aged forest of Mengshan Mountain in northern China is progressive (Gao et al., 2013; 2018). In this study, *P. thunbergii* was used to construct the genetic distance using gene sequencing analysis technology to distinguish close and distant relatives. In addition, chlorophyll (a and b) was measured calorimetrically, which represent primary production capacity and phenolic acids (ferulic acid, vanillic acid and syringic acid) was measured by high-performance liquid chromatography, which are typical plant secondary metabolites to reveal the presence of kin recognition in gymnosperms.

Materials and Methods

Plant Material

The plant used in this experiment was black pine, which is a common species found in artificial forests in northern China. This plant species exhibits rapid growth, long life, complex root system, strong resistance, wide adaptability and requires relatively less soil for growth. Thus, it has high economic value and can be used for greening purpose. The tested black pine trees were wild trees, with an approximate age of 30 years, breast diameter of ca. 15 cm, and height of ~10 m. They were located in the Tianmeng scenic area of Mengshan in Shandong province, China.

Sample Collection

Selected three groups of wild black pines were arranged in a straight line with a ca. 2 m spacing in January 2016. The terrain was flat, and soil conditions were same, with no other woody plants around. A total of five groups of 15 black pines were selected and for each black pine, healthy pine needles were sliced and divided into three groups. All needles were put into labeled and sealed bags for the detection of phenolic acid and chlorophyll content, as well as gene sequencing analysis.

Analytical Methods

Detection of chlorophyll (a and b): Leaves (0.1 g) were measured, sliced and placed in a mortar, followed by the addition of ethanol (10 mL) and homogenized. Then, 5 mL of ethanol was added and the mixture was filtered, and filtrate was diluted to 25 mL with ethanol. The optical density of pigment solution was measured at 649 nm and 665 nm, and the content of chlorophyll was determined (Zhang et al., 2018). This analysis was performed in the Environmental Laboratory of Qingdao Science and Technology Institute.

Determination of phenolic acids (ferulic acid, vanillic acid and syringic acid): After extraction and centrifugation, leaf sample solution was filtered through 0.45 µm filter, and K₂HPO₄ buffer solution (pH 2.5) was used as mobile phase. After ultrasonication for 20 min, samples were analyzed using high-performance liquid chromatography (USA, HPLC Diane Ultimate 3000) equipped with C18 column. UV detector was set at 280 nm to determine the contents of p-hydroxybenzoic acid, vanillic acid, syringic acid, coumaric acid and ferulic acid. This analysis was performed in the Environmental Laboratory of Qingdao Science and Technology Institute.

Gene sequencing analysis: The genomic DNA from pine needle was extracted using plant DNA extraction kit, and three sets of primers were designed as follows: A (FAM fluorescence): Pt26081F/R, Pt79951F/R and Pt107517F/R; B (TET fluorescence): Pt36480F/R, Pt45002F/R and Pt48210F/R C (HEX fluorescence): Pt30204F/R, Pt102584F/R, Pt107148F/R and Pt71936F/R for post-PCR sequencing analysis. These experiments were performed in the Environmental Laboratory of Qingdao Science and Technology Institute.

Statistical Analysis

Excel 2003 and SPSS 17.0 Chinese version were used for processing and one-way analysis of variance was performed.

Results

Grouping of Close and Distant Relatives of *P. thunbergii*

In practice, the middle black pine of the three in a straight line was treated as a marker and the degree of relatives of other two black pines, relative to marker, was measured. Non-marker black pines were divided into two groups, designated as close relatives and distant relatives (Fig. 1).

Differences in Chlorophyll Content between Black Pine Relatives and Distant Relatives

It was found that the content of chlorophyll a and chlorophyll b in pine needles were significantly higher in close relatives than distant one (both $P < 0.05$) (Fig. 2A and B).

Differences in Phenolic Acid Content between Black Pine Relatives and Distant Relatives

It was found that the contents of ferulic acid, vanillic acid and syringic acid in pine needles were significantly ($P < 0.05$) higher in close relatives than distant relatives (Fig. 2C-E).

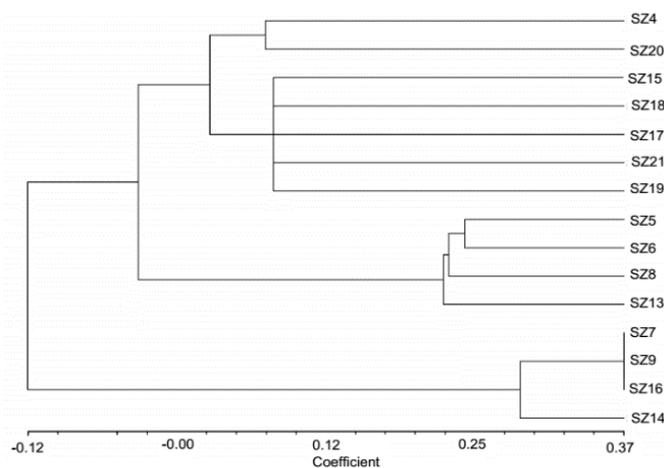


Fig. 1: Genetic distance of tested black pines

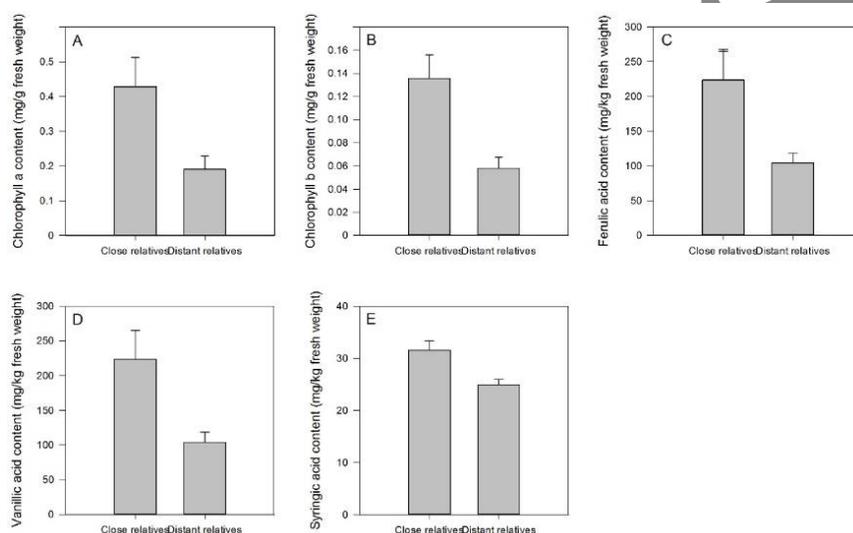


Fig. 2: Differences in chlorophyll and phenolic acid content between close relatives and distant relatives (mean \pm standard error).

A, chlorophyll a; B, chlorophyll b; C, ferulic acid; D, vanillic acid; E, syringic acid

Discussion

There is a large variation in the definition of kinship and the choice of kinship, resulting into three different criteria as follows: 1) same parentage (Bhatt *et al.*, 2011; Semchenko *et al.*, 2014); 2) Genotype (Crepy and Casal, 2015); and 3) Artificial clones (Karban and Shiojiri, 2009; Mercer and Eppley, 2014). In this study, gene sequencing technology was used to distinguish the individuals by genetic distance (Ostrowski *et al.*, 2008). In practice, the middle black pine of the three in a straight line was treated as a marker and the degree of relatives of other two black pines, relative to marker, was measured. Non-marker black pines were divided into two groups, designated as close relatives and distant relatives at a genetic distance of 0.03 (Fig. 1).

In recent years, researchers have reported that when *Rosmarinus officinalis* co-existed with same species, phenolic acids content in the leaves was significantly

increased (Ormeño *et al.*, 2007). Similarly, when *Centaurea maculosa* co-existed with same species, secondary metabolic processes, such as phenolic synthesis and protease inhibitor synthesis in leaves, were promoted (Broz *et al.*, 2010). In addition, when *Arabidopsis thaliana* co-existed with distant relatives, the expression of disease-related genes was up-regulated in the roots (Biedrzycki *et al.*, 2011) and the number of disease-deficient proteins in root exudates was significantly increased (Badri *et al.*, 2012). These studies confirmed that when plants co-existed with same species, more energy was utilized for secondary metabolism, resulting in higher production of phenolic acids and antioxidant enzymes in the plants to protect against invasion of animals and microbes. Meanwhile, the input of primary metabolism was reduced in many processes (*e.g.*, leaf and root formation) to reduce the competition of same species for external nutrients and enhance the resistance to external environmental stress (Lin *et al.*, 2015).

In this study, it was found that when black pines co-existed with same species, the content of chlorophyll a (Fig. 2A), chlorophyll b (Fig. 2B), ferulic acid (Fig. 2C), vanillic acid (Fig. 2D) and syringic acid (Fig. 2E) were increased in pine needles. In addition, these contents were significantly higher in close relative group compared to that of distant relative group.

Since the tested black pine plants in this study were naturally grown, some environmental variables should be considered. However, given the existing literature available, kin recognition could explain the differences in the primary metabolites production and secondary metabolite distribution in black pine needles in close and distant relatives. It has been found that the population of *P. thunbergii* in the middle-aged forest of Mengshan Mountain in northern China is progressive (Gao et al., 2013). The latest research found that the pine needle litter of *P. thunbergii* contains allelochemicals (Zhang et al., 2018). This study was the first to discover and confirm the existence of kin recognition in the black pine, and also is the second reported kin recognition case in gymnosperm worldwide. This study provides a new explanation for the population adaptation and niche of *P. thunbergii* in northern China, that is, *P. thunbergii* is a kinship-promoting type.

Conclusion

Based on chlorophyll a and b (represents the primary production capacity of plants), and phenolic acids (typical plant secondary metabolites), under natural conditions, *P. thunbergii* could identify the distant relatives of neighbors and adjust physiological strategies accordingly. Furthermore, the population of *P. thunbergii* in the middle-aged forest of Mengshan Mountain in northern China is progressive, which provides a basis for *P. thunbergii* population adaptation in the northern China.

Acknowledgements

Funded by the National Natural Science Foundation of China, NO. 41401329 and Fund of Shandong Provincial Key Laboratory of Water and Soil Conservation and Environmental Protection, Linyi University, NO. STKF201906.

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